αMSH in fish
Functions in stress responses and skin colour change

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Functions in stress responses and skin colour change

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Aan mijn ouders

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Chapter 1

Introduction

The ability to adapt to changes in the environment is a key process in the survival of all living beings. In this thesis, two of these processes will be studied in more detail: first, adaptation to the environment by a change of the body colour and second, adaptation to environmental challenges that can be defined as stressors and may affect the physiological state of an animal. Fish are ideal models to study both processes due to their ability to change skin colour rapidly and also because the aquatic environment allows the experimenter for instance to easily manipulate the water quality, and by doing so, animals can be exposed to a variety of well-defined stressors with relative ease. Interestingly, several hormones are known to act in both these processes. The main hormone of interest in this thesis will be α-melanophore-stimulating hormone (αMSH), described as a hormone involved in skin colour regulation as well as in the response to stress.

Animal pigmentation

Throughout the animal kingdom, an astounding richness of colours can be observed. Besides the mere beauty that we appreciate as human beings, these colour patterns serve many different functions. Animals use body colour for camouflage, to facilitate predation or to avoid predators; to warn predators of a bad taste or to mimic the colour patterns of distasteful or poisonous species; to recognize members of their own species either to warn them to stay away from their territory or to invite them into social and sexual interactions. Body colours result from the presence of different types of pigment cells in various combinations. Skin pigment cells, or chromatophores, are subdivided into at least six different types (Fujii, 2000): melanophores (black or brown, depending on the type of pigment), xanthophores (yellow), erythrophores (red), cyanophores (blue), leucophores (whitish) and iridophores (iridiscent).

In homeothermic animals such as mammals or birds, coat or feather colour can change via a complete renewal of these skin derivatives, for example to match the change of seasons. Human hair does not change colour throughout life until old age inhibits the pigmentation and the colour turns grey. Also, skin colour in humans can only darken or lighten and this depends on the amount of UV radiation received from the sun. So, in mammals and in birds, body colour change is a time-consuming process that may take days to weeks.

A variety of poikilothermic animals change their skin colour rapidly, within minutes. The chameleon is proverbially known for this feature. These rapid colour changes (called physiological colour changes; Bagnara and Hadley, 1973; Fujii, 1993, 2000) are possible because of the motile activity of the pigment granules within the chromatophores. Melanophores have been studied most extensively in fish and amphibians alike. The granules of pigment within these cells are called melanin granules. In mammals and birds, these melanin granules contain eumelanin, a black pigment, and phaeomelanin, which has a brown-red colour. In poikilothermic animals only the eumelanin is present (Bagnara and Hadley, 1973). Melanophores have long dendritic extensions within which the

melanin granules are transported to give the cell a visible shape ranging from a dark, stellar or dispersed appearance to a light, concentrated or aggregated appearance (see Figure 1). A simple method to identify the degree of dispersion of melanophores was originally described by Hogben and Slome (1931; Figure 1). Melanophores are classified into five different stages ranging from completely aggregated (melanophore index; MI 1) to completely dispersed (MI 5). This classification provides the experimenter with a fast and simple method of scoring the effect of dispersing or aggregating agents on melanophores.

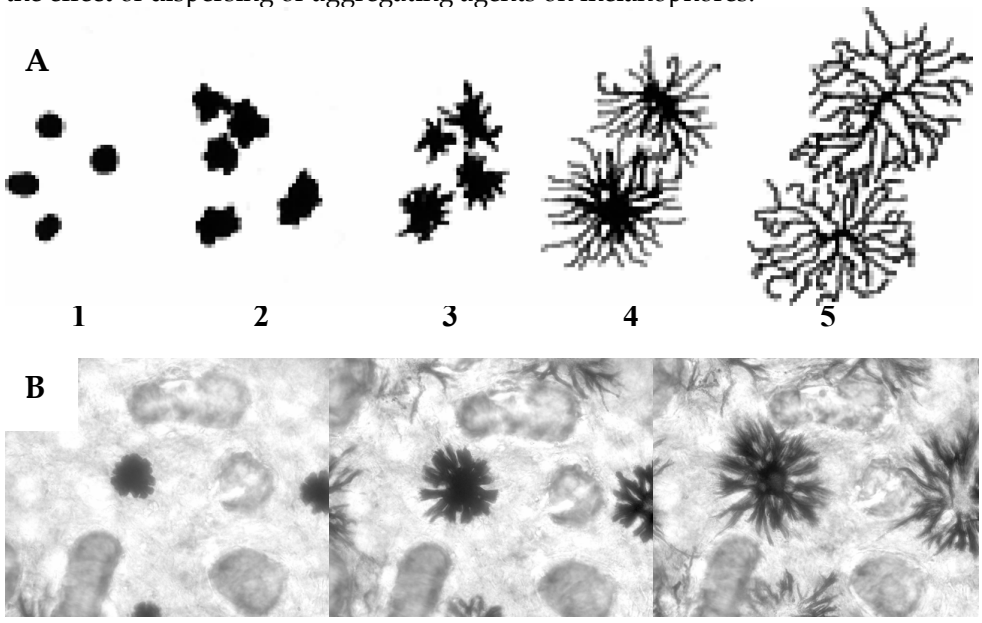

*Figure 1- A) Melanophore index (MI) after Hogben and Slome (1931) where 1 indicates a completely aggregated and 5 a completely dispersed melanophore; B) tilapia (*Oreochromis mossambicus*) scale melanophore in corresponding MI stages 2, 3 and 4 (from left to right). This scale was treated with a 60 mM K^+ solution, causing aggregation of the melanophores from stages 4 / 5(right image) to stages 1 / 2 (left image).*

More recent methods to estimate the pigmentation state of an animal include the use of photoelectric recordings (Hayashi et al., 1996), determination of melanin content, and morphometrical techniques facilitated by modern computer software, which allow determination of the exact size and numerical density of melanophores (Sugimoto et al., 2000).

The regulation of fish melanophore responsiveness to different substances and conditions has been studied extensively in a number of species, as reviewed by Fujii (2000). The diagram shown in figure 2 has been modified after this author and shows a simplified model for the regulation of dispersion and aggregation of melanin granules in a fish melanophore.

Two hormones in particular have been implicated in the endocrine control of skin pigmentation in lower vertebrates: melanin concentrating hormone (MCH), which is released from the hypothalamus and has an aggregating effect on the melanin granules in the melanophore, and αMSH that is released by the pituitary gland into the bloodstream and has a dispersing effect on the melanin granules.

Catecholamines (noradrenalin; NE, released via nerve fibres, and adrenaline; Epi, released from the chromaffin cells in the head kidney) act as neurotransmitters and can have either a dispersing or aggregating effect, depending on the receptor subtype they activate. Stimulation of α-adrenoreceptors causes aggregation of melanin, while β-adrenoceptor stimulation evokes melanin dispersion.

Stress responses

Stress can be defined as a condition in which the dynamic equilibrium of an organism is threatened or disturbed by internal or external stimuli called stressors (Wendelaar Bonga, 1997).

Through the stress response, an animal tries to cope with a stressor by altering its many physiological activities. In line with research performed in other vertebrate species, teleost fish can show acute and chronic stress responses, depending on the intensity and duration of the stressor, as described below.

The acute stress response, as the name implies, can occur after sudden exposure to pronounced changes such as elevated concentrations of toxins, fast temperature drops, hypoxia, salinity changes, capture or handling, and involves a fast increase (within seconds of the stressor being noticed) in catecholamine levels to increase oxygen uptake and transport via the blood, as well as mobilization of energy substrates (Perry and Bernier, 1999; Reid et al., 1998; Wendelaar Bonga, 1997). This increase of catecholamine levels is the result of the activation of the hypothalamic-sympathetic-chromaffin-cell (HSC) axis. After the brain has processed a stressful stimulus, the hypothalamus sends a signal to the chromaffin cells in the head kidney via sympathetic nervous pathways to release the catecholamines. A second axis is also activated: the hypothalamic-pituitary-interrenal (HPI) axis, which increases the release of cortisol from the interrenal cells of the head kidney within a few minutes after exposure to the acute stressor (Figure 3). Cortisol is generally accepted as the main stress hormone, causing a variety of physiological changes to counter the stressor (Mommsen et al., 1999; Tort, 1998; Wendelaar Bonga, 1997).

The second type, the chronic stress response, occurs when stressors persist, such as during long term exposure to toxins, acidified water conditions and prolonged social stress. Both cortisol and catecholamine levels will not reach levels as high as during acute stress but will remain elevated above basal levels.

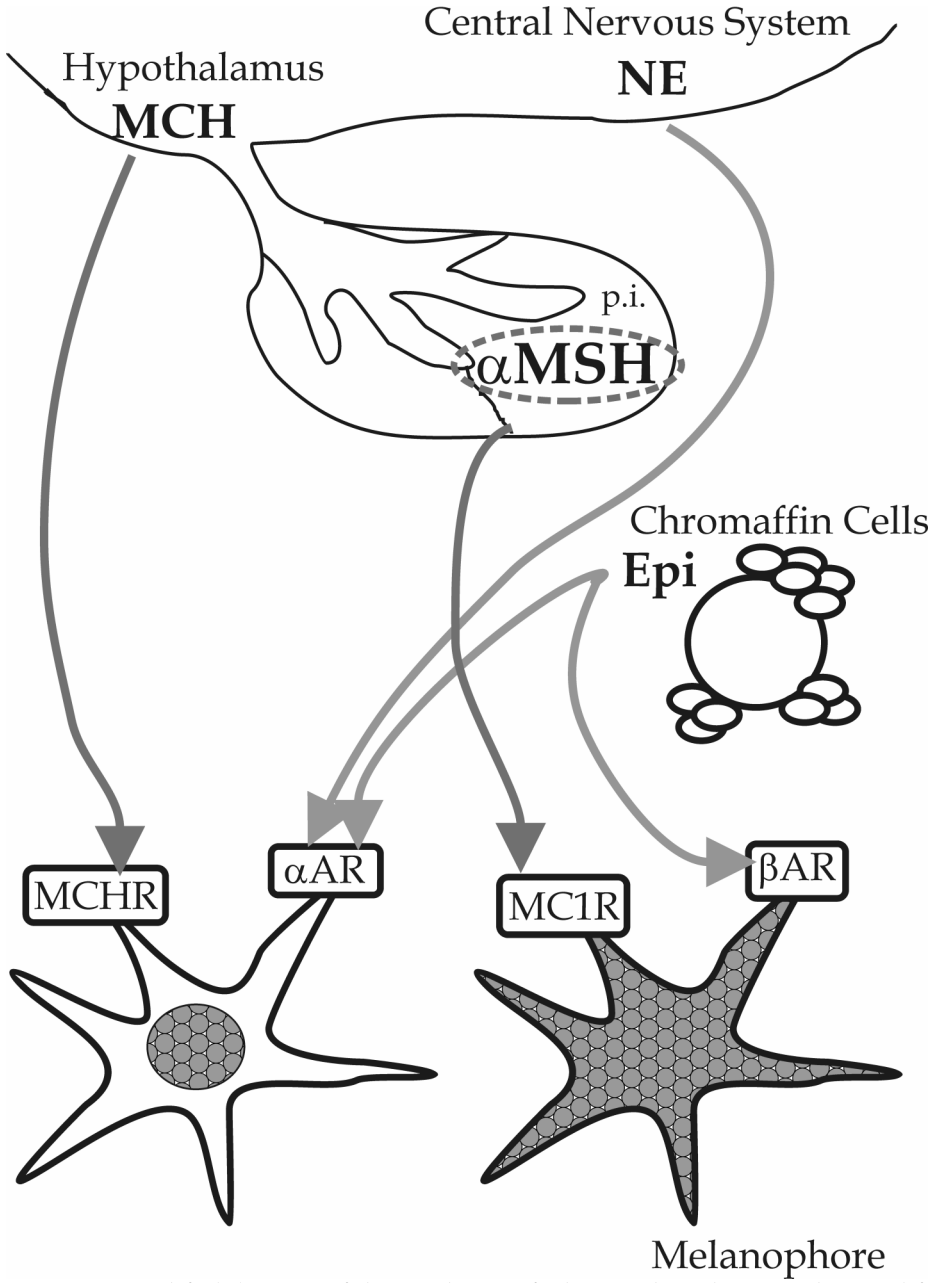

Figure 2- a simplified diagram of the regulation of teleost melanophore motility modified after Fujii (2000). MCHR=MCH receptor, αAR= α-adrenoreceptor, MC1R= melanocortin-1 receptor, βAR= β-adrenoreceptor, MCH=melanophore concentrating hormone, NE=noradrenalin, Epi=adrenaline, αMSH=α-melanophore-stimulating hormone and p.i.= pars intermedia; the area of the pituitary gland where melanotrope cells are located.

The release of cortisol is primarily under control of adrenocorticotropic hormone; ACTH (see Figure 3, curved arrows). However, αMSH has also been reported to have corticotropic potencies in the tilapia, *Oreochromis mossambicus*, in combination with β-endorphin (Balm et al., 1995; Lamers et al., 1992). These peptides are derived from the precursor molecule proopiomelanocortin (POMC). Upon synthesis, this peptide is differentially cleaved into ACTH and opioid β-endorphin (in the corticotropic cells in the pars distalis of the pituitary gland) or into αMSH (in the melanotrope cells of the pars intermedia of the pituitary gland). N-acetylated β-endorphin (the non-opioid form of the peptide) and two other MSH forms (βMSH and γMSH) are also derived from the POMC precursor hormone (Klovins et al., 2004).

The release of αMSH in fish is under multifactorial hypothalamic control. MCH and dopamine (DA) have an inhibitory effect on the release of αMSH in several species of fish (Barber et al., 1987; Green and Baker, 1991; Lamers et al., 1997; Omeljanuk et al., 1989), while thyrotropin-releasing hormone (TRH) and corticotropin-releasing hormone (CRH) stimulate the release of αMSH from the pituitary gland (van den Burg et al., 2003; Rottlant et al., 2001).

After synthesis of αMSH by the melanotrope cells, acetylation of the peptide can occur prior to its release. αMSH can be found in three acetylation states: des-acetylated αMSH (no acetyl-group) and mono or di-acetylated αMSH (one or two acetyl-groups respectively; Arends et al., 2000; Dores et al., 1993; van Strien et al., 1995). The bioactivity of peptides in general can vary depending on the degree of acetylation, although the effects are species-specific (Arends et al., 2000; Lamers et al., 1992, 1994; Mountjoy et al., 1999). Another factor affecting the bioactivity of peptides is the affinity profile of the receptor for different isoforms of a peptide.

Recent research has identified 5 types of G-protein-coupled 7-transmembrane receptors that respond to POMC-derived peptides. These melanocortin receptors (MCRs), designated MC1R to MC5R, were first identified in mammals but have now also been identified in fish and show remarkable amino acid sequence homology between mammals, birds and fish (Logan et al., 2003; Schiöth et al., 2003). In mammals, MC1R is expressed mainly in melanophores but also in leukocytes and other peripheral cells. MC2R is expressed solely in the adrenal cortex, while MC3R and MC4R are involved in energy homeostasis and are expressed throughout the brain, particularly in the hypothalamus. MC5R is expressed in several peripheral tissues (Klovins et al., 2004). Research in fish indicates that the expression patterns of these receptors differ somewhat from the mammalian patterns. In the pufferfish (*Takifugu rubripes*), the head kidney expresses MC2R, MC4R and MC5R, although this may relate to the complex cellular composition of the fish head kidney that not only contains chromaffin cells and interrenal cells but also cells that are part of the immune system. The affinity of these receptors to the different POMC-derived peptides varies per receptor type. There are also great differences in affinity of the different receptor types between humans and fish and even between various

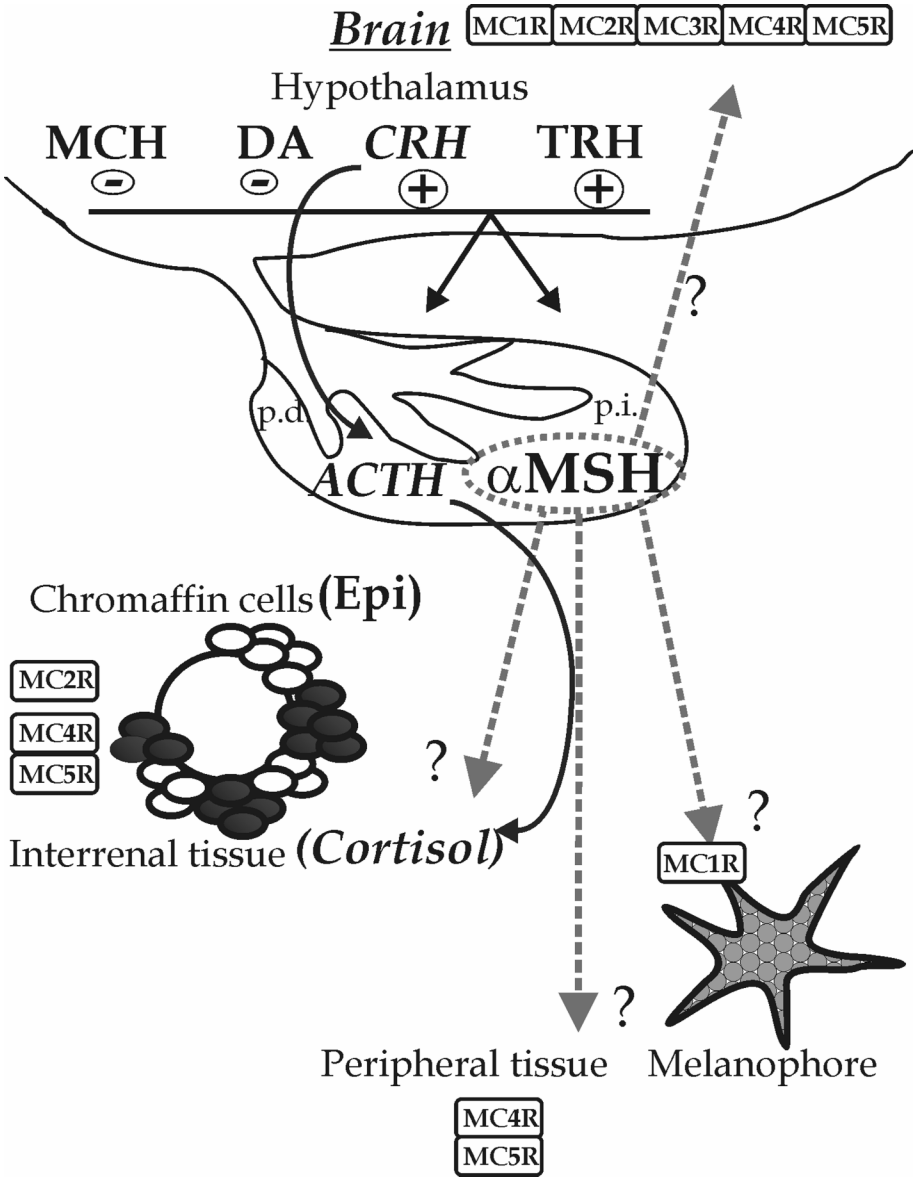

Figure 3- the stress axis in fish. The hypothalamic-sympathic-chromaffin axis comprises a direct stimulation of chromaffin cells in the head kidney to release catecholamines (e.g. adrenaline; Epi), while the hypothalamic-pituitary-interrenal axis constitutes of hypothalamic factors that stimulate (+) or inhibit (-) the pituitary gland to release (amongst other factors) ACTH or αMSH, which can stimulate the interrenal cells in the head kidney to release cortisol (modified after Wendelaar Bonga, 1997). The bioactivity of αMSH and ACTH depends on their binding to the different melanocortin receptors (MC#R; Klovins et al., 2004).

fish species (Haitina et al., 2004; Klovins et al., 2004). To date, we can assume that all fish MCR subtypes, except perhaps the MC2R, which appears to be ACTH specific (Metz and Flik, submitted), bind αMSH.

Interactions between pigment changes and the stress response

The agitation following an encounter with a stressor often results in pigmentation changes. While humans blush or pale, fish can choose to adopt an aggressive pigmentation pattern or perhaps a more submissive hue. These changes often occur during encounters between animals of the same species challenging each others authority (Fox et al., 1997).

Several of the hormones mentioned above are involved in pigmentation changes as well as in the response to stressors. αMSH and MCH have antagonistic effects on the migration of melanin granules within the melanophore (Figure 2; Burton and Vokey, 2000; Logan et al., 2003; Sumpter et al., 1984). Within the HPI axis, MCH has an inhibitory effect on the release of αMSH (Figure 3; Barber et al., 1987; Gröneveld et al., 1995), and the secretion of MCH itself is also directly influenced by stressors (Green et al., 1991; Green and Baker, 1991).

Adrenaline and noradrenalin have a similar antagonistic relationship as MCH and αMSH on pigmentation. Binding of catecholamines to α-adrenoreceptors will result in aggregation while binding to $\beta_{(2)}$-subtype adrenoreceptors will cause dispersion of melanin granules in fish (Aspengren et al., 2003; Fujii, 2000; Mårtensson et al., 1999; Stone and Chavin, 1974). Catecholamines are also the most important agents in the adrenergic or nervous part of the acute stress response (the HSC-axis).

Functions of αMSH – Aim and outline of this thesis

The involvement of αMSH in melanin migration and the rapid colour changes that can be seen in lower vertebrates have been documented extensively. These findings, together with various reports on elevations of the plasma αMSH concentration during stress and the direct effects of αMSH within the HPI-axis, point to a possible interaction between pigmentation control and the stress response (Arends et al., 1999; van Eys and Peters, 1981; Fernandez and Bagnara, 1991; Fujii, 2000; Lamers et al., 1991; Lindley et al., 1990; Rottlant et al., 2000; Sugimoto, 2002; Sumpter et al., 1984, 1985; Zhu and Thomas, 1996). This relationship is the focus point of this thesis; the functions of αMSH both during background adaptation and stressful situations will be examined in two species of fish as described below.

In the first chapters, we studied the role of αMSH in the red porgy, *Pagrus pagrus*, as part of the EU-project "Colored". This sparid seawater species is a candidate for aquaculture. Its close relatives, the gilthead sea bream and the sea bass are already cultured successfully. An important problem encountered during rearing of red porgy is the marked darkening of the body that occurs

under common culture conditions, which are often stressful. This body darkening results in a dramatic loss of market value. While wild red porgy exhibit an attractive pink and silvery body colour, cultured fish generally show a dark grey body colour. This species is encountered mainly in the Atlantic Ocean and the Mediterranean Sea. This part of our research was therefore conducted in cooperation with marine institutes in Gran Canaria (Spain, on wild-captured red porgy) and Crete (Greece, on cultured red porgy).

In Chapter 2, the release of αMSH was studied in cultured red porgy. The effects of MCH, DA, TRH and CRH on the release were determined and a possible differential release of the three αMSH isoforms was investigated.

In Chapter 3, the effects of background adaptation on the response to chronic crowding stress, a situation not uncommon in aquaculture conditions, were investigated. Also, two other aquaculture-related conditions possibly influencing the stress response or the body pigmentation were evaluated, namely the illumination spectrum and the intensity of illumination.

In Chapter 4, red porgy that had previously been allowed to adapt to different backgrounds, were subjected to a severe acute stress: a combination of netting and air exposure for five minutes. The response was followed for 24h.

Mozambique tilapia, *O. mossambicus*, is a freshwater species that is successfully cultured worldwide due to its sturdiness and high protein content. The ability of this species to adapt its skin colour to the background, combined with extensive knowledge on the stress response makes it an ideal model for fundamental research on the role of αMSH in background adaptation and in the stress response. Previous research has indicated that αMSH causes a darkening of the body and that it can exert corticotropic effects during chronic low pH stress (van Eys and Peters, 1981; Foo and Lam, 1993; Ginneken et al., 1997; Gröneveld et al., 1995; Hulscher-Emeis, 1992; Lamers et al., 1994; Quabius et al., 2000; Vijayan et al., 1997).

In Chapter 5, tilapia were kept on three different backgrounds and were exposed to low water pH, either during background adaptation or after 25 days of undisturbed adaptation to the different backgrounds.

Chapter 6 deals with the *in vitro* responses of tilapia scale melanophores after prolonged background adaptation to des-, mono- and di-acetylated αMSH. In this chapter the sequence of the MC1R in tilapia is presented and the expression of this receptor on the different backgrounds is investigated.

In Chapter 7 the significance of αMSH in the regulation of pigmentation in teleost species and the importance of its role in the stress response will be discussed. Results are compared with current knowledge on the functions of the pleiotrope hormone αMSH in fish.

Chapter 2

Differential release of α-Melanophore Stimulating Hormone isoforms by the pituitary gland of red porgy, *Pagrus pagrus*

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General and Comparative Endocrinology (2004), 135: pp. 126-133

Abstract

The best known actions of the pleiotrope α-melanophore-stimulating hormone (αMSH) are skin pigment regulation and corticotrope actions in the response to chronic stress. Stress-induced and enhanced release of αMSH may therefore influence skin pigmentation and stress physiology simultaneously. The release of αMSH is under multiple control by hypothalamic hormones and neurotransmitters. Thyrotropin-releasing hormone (TRH), corticotropin-releasing hormone (CRH), melanophore-concentrating hormone (MCH) and dopamine (DA) have been tested in a superfusion set up for their potential to regulate αMSH release from the pituitary gland of red porgy, *Pagrus pagrus*, *in vitro*. The release of αMSH was stimulated by TRH and CRH, and was inhibited by MCH and DA. During unstimulated (basal) release, mono-acetylated αMSH was the dominant form. During superfusion with secretagogues, we found that independent of their inhibitory or stimulatory capacity, isoform frequency did not change. MSH-isoform ratios were similar for all substances used, except that both the inhibitory and the stimulatory factors increased the percentage of di-acetylated αMSH at low concentrations (10^{-11} M) when compared to their effects at high concentrations (10^{-7} M).

Introduction

Alpha-melanophore-stimulating hormone (αMSH) is pivotal in the regulation of skin pigmentation. It was the first hormone identified as a colour changing agent in poikilothermic species (Bagnara and Hadley, 1973). αMSH stimulates melanin synthesis and melanosome movement within the melanophores (Castrucci et al., 1997). Furthermore, on the longer term it increases both melanophore number and size, resulting in darkening of the skin. In addition to this classic pigmentation function of the hormone, there is evidence in tilapia that αMSH plays a role as a corticotrope hormone in the chronic stress response (Lamers et al., 1992; Wendelaar Bonga, 1997). The corticotrope action was confirmed for other species as plasma αMSH levels increase following air exposure of sea bream (Arends et al., 1999) and brown trout (Sumpter et al., 1985). The corticotrope activity of αMSH may be potentiated by β-endorphin (Balm et al., 1995).

Red porgy (*Pagrus pagrus*) is a species rather new in aquaculture. A main problem in moving onwards to grand scale production is the skin darkening that occurs in cultured fish (Kentouri et al., 1995). Wild fish have a red and silvery skin colour, which turns into a greyish dark within a number of weeks of captivity. Intensive rearing includes a number of stressful stimuli for fish, including handling stress and confinement. It was found for gilthead sea bream that these stressors increased not only cortisol levels, but also αMSH levels in blood plasma (Arends et al., 1999). Moreover, wild adult red porgy are often found at depths from 150 to 250 m, which implies that normal aquaculture conditions involve a dramatic change in environmental variables such as light and pressure. Thus, the greyish skin colour of red porgy in captivity may be related to stress and therefore this species was considered a suitable model to study the relationship between the pigment-regulatory function of αMSH and its possible involvement in the stress response.

In teleosts, αMSH is produced in the pars intermedia of the pituitary gland. It is derived from the precursor hormone proopiomelanocortin (POMC). POMC is found in the pituitary gland, and is differentially cleaved into a number of peptide hormones depending on the cell type it is produced in (Lamers et al., 1991; Steveson et al., 1996). There are various hypothalamic regulatory factors that control the release of αMSH from the pituitary gland (Wendelaar Bonga, 1997). Release can be stimulated *in vitro* by thyrotropin-releasing-hormone (TRH; van den Burg et al., 2003; Lamers et al., 1991; Rottlant et al., 2000) and corticotropin-releasing hormone (CRH; Rottlant et al., 2001). In tilapia, CRH induces a strong release of αMSH, although rather pharmacological concentrations (μM) are required *in vitro* to obtain this result. TRH is a mild stimulator and operates *in vitro* at presumed physiological (pM - nM) concentrations. In chronically stressed fish the pituitary αMSH cells show hyperplasia and these cells become more sensitive to TRH specifically (Lamers et

al., 1994). αMSH is under inhibitory control of melanin-concentrating hormone (MCH). This neuropeptide was previously identified as an αMSH antagonist since it can aggregate melanophores (see Burton et al., 2000, for references), but later it was shown to antagonise αMSH by an inhibitory control at the pituitary level (Barber et al., 1987), an effect that increased during stress (Green and Baker., 1991). Another inhibitory factor of αMSH release is dopamine (DA; Lamers et al., 1991, 1997; Omeljanuk et al., 1989). Inhibitory effects are evoked via a D2-subtype receptor. Interestingly, in chronically stressed tilapia a stimulatory D1-type receptor is expressed and allows for stimulatory actions by picomolar concentrations of DA on the αMSH cells.

Peptides are often posttranslationally modified by glycosylation, amidation or acetylation. αMSH is found in three different isoforms: des-, mono- and di-acetylated αMSH (Arends et al., 2000; Lamers et al., 1991; van Strien et al., 1995). In mammals, as in most other species where acetylation occurs, the major form is diacetyl αMSH (Dores et al., 1993; Keller et al., 1994). In a number of fish species, however, the dominant form is monoacetyl αMSH (Dores et al., 1993; Arends et al., 2000). Since acetylation can modify the activity of the peptides (Keller et al., 1994), it is quite possible that differently acetylated isoforms exhibit different properties.

The aims of the present study were: 1) to determine the effect of different hypothalamic messengers on the release of αMSH from the pituitary gland of cultured red porgy *in vitro*, namely TRH, CRH, MCH or DA; they were tested in a concentration-response fashion in a superfusion set up; 2) to identify the acetylation profile of αMSH in red porgy by means of high performance liquid chromatography (HPLC), and 3) to see whether the ratio of the different isoforms is influenced by different hypothalamic messengers.

Materials and Methods

Fish maintenance

Fish were kept in 500L circular polyester tanks filled with sea water, continuously replaced with a mixture of 1/3 fresh seawater and 2/3 recycled water in the Institute for Marine Biology, Crete. At the start of the experiments, 20-30 fish were distributed over five tanks. Water temperature and oxygen content were monitored daily (20.1 ± 0.08 °C, 5.25 ± 0.08 mg/l respectively). Fish were fed commercial pellets (Biomar Hellenic S.A., Velestino, Greece) by self feeders.

Superfusion technique

Upon sampling, 8 fish per superfusion experiment were deeply anaesthetized in 0.2% phenoxyethanol and weighed. Blood was drawn from the caudal veins, with syringes containing 35µl of 2% Na-EDTA to prevent clotting, and 100µl (corresponding with 1 TIU) of aprotinin to prevent proteolysis. The

blood was spun at 4°C for 5 min at 1500 rpm, after which the supernatant plasma was stored in Eppendorf vials and quickly frozen. The fish were decapitated and the pituitaries dissected. The latter were subsequently placed in wells containing 1ml of superfusion buffer and after all pituitaries had been dissected (usually within 30 minutes) they were transferred to superfusion chambers. On the bottom of the chambers a cheese-cloth filter was placed to avoid loss of the tissues into the system, and the pituitaries were superfused with a HEPES (15mM)/Tris-buffered Ringer's solution (pH 7.4) containing NaCl (152mM), KCl (2mM), $CaCl_2.2H_2O$ (2mM), 0.25% (w/v) glucose, 0.03% (w/v) bovine serum albumin and 1mg/l ascorbic acid. The temperature of the buffer was adjusted to match the temperature of the water the fish were taken from. Aerated medium was pumped through the chambers at 30µl/min by a Watson Marlow 503U multichannel peristaltic pump (Smith and Nephew Watson-Marlow, Falmouth, Cornwall, U.K.). After three hours of superfusion, the release rate of αMSH was stable. At this time, medium supplemented with TRH, CRH, DA or MCH was administered for 30 minutes, in concentrations from 10^{-7} to 10^{-11} M. Release was allowed to return to basal following application of the secretagogues. After five hours of superfusion (90 minutes after secretagogue application) the pituitary glands were superfused with medium containing 60mM K^+ to depolarise the cell and induce release of stored αMSH. Fractions were collected with an Isco fraction collector every 15 minutes, except during the 30-min pulse with secretagogue or the 15-min control pulse with 60mM K^+, when fractions were collected every five minutes. The whole experiment lasted six hours. Collected fractions were stored at –20 °C until further analysis. Basal, unstimulated release was calculated as the mean rate of release between 120 and 180 minutes, and set at 100%. Stimulation or inhibition of αMSH release was expressed as the percentage relative to basal levels.

Radioimmunoassays

The αMSH concentration in the superfusion fractions was determined according to Arends et al. (1999). The antiserum used for the αMSH radio immuno assay (RIA) cross-reacts for 100% with des-, mono- and di-acetyl αMSH (Vaudry et al., 1978), and was used in a final dilution of 1:60,000. Immunocomplexes were precipitated by 7.5% (w/v) polyethyleneglycol and 2.5 % (w/v) bovine serum albumin (van Zoest et al., 1989). The detection limit was 0.63 pg/25µl of sample. Radioactivity was quantified using a Cobra II γ-counter (Packard Instruments, Boston, USA).

To validate the assay for red porgy αMSH, serial dilutions (1:1) were made in assay buffer from superfusate with a measured high level of αMSH. The displacement of radiolabelled αMSH was plotted for the superfusate and the synthetic standard αMSH.

Reversed-Phase HPLC technique

To separate the different isoforms of αMSH, reversed-phase HPLC was used as described by Arends et al. (2000). First, a mixture of synthetic des-, mono- and diacetyl αMSH (Sigma-Aldrich, St. Louis, USA) was separated on a Pharmacia μRPC C2/C18 sc 2.1/10 column with ddH_2O/0.1% trifluoracetic acid (TFA) as equalibration eluent and a gradient of acetronitril/0.1% TFA from 0-100% as secondary eluent to determine the elution time of each isoform and subsequently, the superfusates were fractionated following this protocol. One-minute fractions were collected and the αMSH concentration in these fractions was determined by RIA. To calculate the amount of des-, mono- and diacetyl αMSH, the area under the curve was measured for each form. The sum of the amounts of des-, mono- and diacetyl αMSH was set at 100%. The amount of each individual form was expressed as a part of this 100%.

Samples subjected to RP-HPLC were selected on the basis of their αMSH concentration as determined by RIA, to secure that sufficient αMSH was present for detection after separation. Only superfusate fractions from the highest and lowest concentrations of secretagogue tested (10^{-7} and 10^{-11}) were selected. The goal of this experiment was to compare the effect of high and low concentrations of secretagogue on the ratio of αMSH isoforms released from the pituitary gland.

Data analysis

Results are given as means ± SEM, and the number of pituitaries used for each data point varied between N=4 and N=6. Concentration-effect curves were fitted using a non-linear data analysis program (SigmaPlot 5.00, SPSS Inc., Chicago, USA). The relative amounts of αMSH isoforms released by the different secretagogues and the different concentrations used, were tested for significance by two-way analysis of variance (ANOVA). Also the differences within treatments were tested by this procedure. Post-hoc testing was done by Tukey HSD. Correlations between the types of secretagogues, or the different concentrations of these substances, and the relative amounts of des-, mono- or diacetylated αMSH were assessed by use of the Pearson Correlation test. These analyses were all performed using SPSS 11.5 for Windows (SPSS Inc.)

Results

Regression analysis showed strict parallelism between the fits for the standard curve and the dilutions of superfusate obtained from red porgy pituitary gland ($P<0.0001$; $r^2 > 0.97$); the slopes of the linear parts of the curves were identical ($P<0.001$). Similar results were obtained for αMSH in red porgy plasma and pituitary extracts (data not shown).

CRH stimulated αMSH release from red porgy pituitary gland only at 10^{-7} M, where a 180% stimulation was observed (Figure 1A; basal release -dashed line- is designated 100%, curve fit $r^2=0.85$). All other concentrations tested did not

affect release. Therefore, an EC_{50} for CRH (defined as the concentration of CRH required for half-maximal response) could not be calculated with the data available.

TRH stimulated the release of αMSH in a concentration-dependent fashion at all concentrations above 10^{-10} M (Figure 1B, fit r^2=0.99). Low concentrations of 10^{-11} and 10^{-10} M, which are close to physiological levels of TRH (Lamers et al., 1994), did not influence the release, whereas concentrations of 10^{-8} and 10^{-7} M TRH stimulated release by about 225%. The estimated EC_{50} for TRH was 7.4 x10^{-10} M.

DA inhibited the release of αMSH to 45 % of basal release at concentrations of 10^{-10} M and higher (Figure 1C, fit r^2=0.99). The calculated IC_{50} value for DA was 3.1 x10^{-11} M.

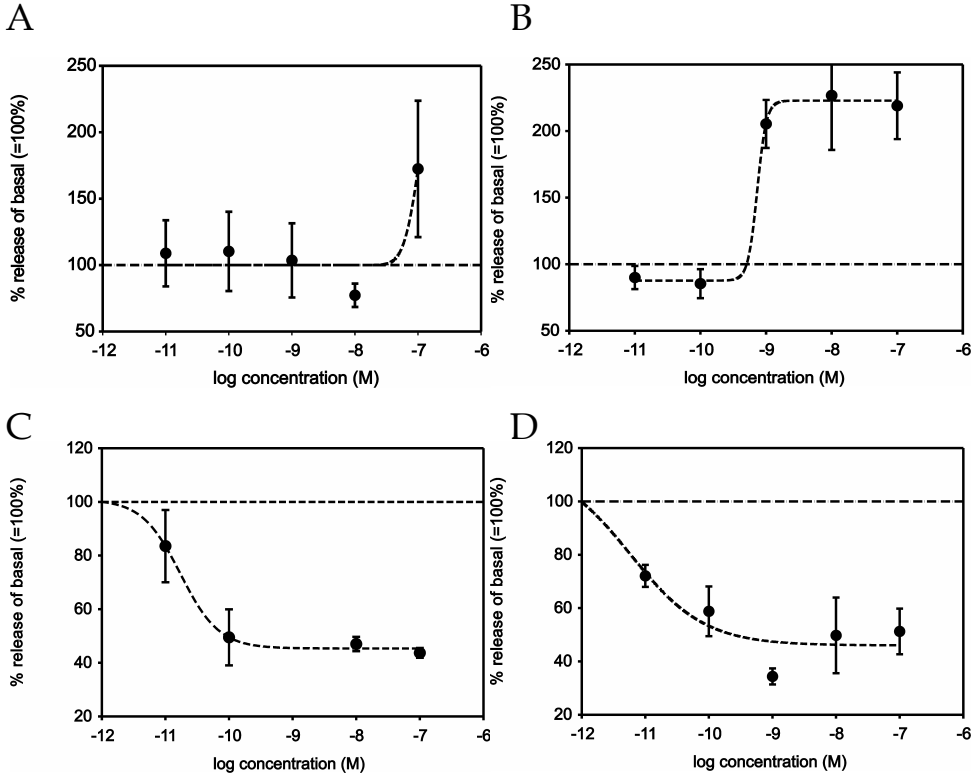

Figure 1- concentration-response curves for CRH (A, curve fit was r^2=0.85), TRH (B, fit r^2=0.99), DA (C, fit r^2=0.99) and MCH (D, fit r^2=0.91) tested on red porgy pituitary glands superfused in vitro *(dotted lines). Data represent means ± SEM (N=4-6). Basal release (dashed line) was designated 100%.*

MCH inhibited αMSH release at all concentrations tested, although inhibition decreased from 10^{-9} M and lower (Figure 1D, fit r^2=0.91). The release

was reduced to 45% of basal release maximally, indicating a partial inhibition of release. The IC_{50} for MCH was 5.9 $x10^{-12}$ M.

Figure 2 shows the isoforms of αMSH released by red porgy pituitary gland as analysed by RP-HPLC. Three isoforms were discerned on the basis of coelution with commercial isoforms: desacetyl αMSH, monoacetyl αMSH (largest peak), and diacetyl αMSH.

The RP-HPLC results for a selection of the superfusates are shown in Table 1. In all cases, both during basal release and after stimulation / inhibition by the secretagogues tested, monoacetyl αMSH was the dominant form released. When comparing all treatments for their relative amount of desacetyl, monoacetyl or diacetyl αMSH we found no significant differences between the different treatments. When comparing the ratios within a treatment, however, we found that in nearly all cases the relative amount of monoacetyl αMSH was significantly higher than the relative amount of diacetyl αMSH. In most cases, the relative amount of monoacetyl αMSH was also significantly higher than the relative amount of desacetyl αMSH (see Table 1). In none of the treatments the relative amount of desacetyl αMSH was different from that of diacetyl αMSH.

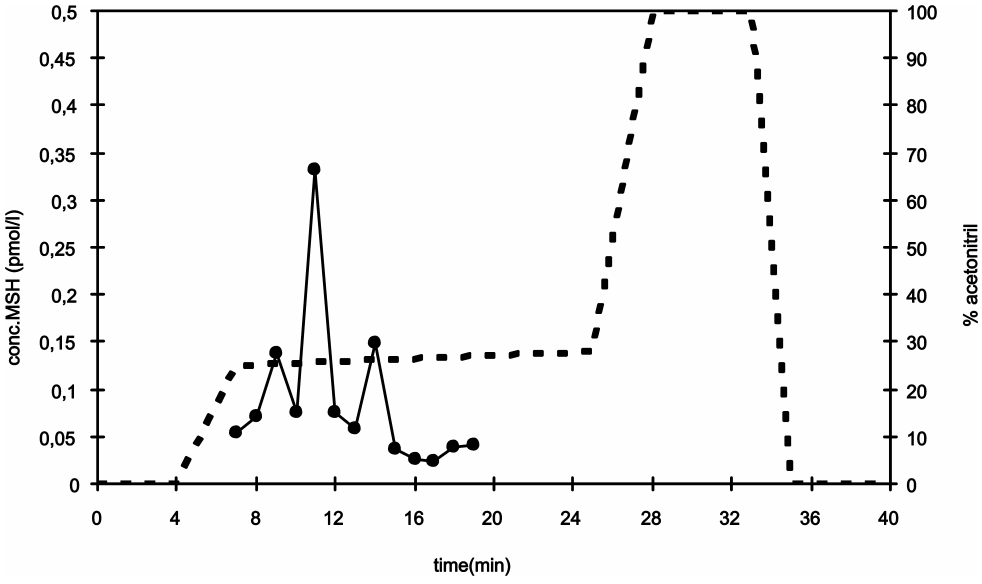

Figure 2- a typical example of a RP-HPLC immunochromatogram for αMSH isoforms (The figure shows the elution pattern of products released by a pituitary gland stimulated with 10^{-11} M TRH.). In this graph, the first peak represents desacetyl αMSH, the second is monoacetyl αMSH and the third is diacetyl αMSH, based on coelutions with synthetic peptides. The dotted line represents the concentration of elution buffer (0.1% TFA in acetonitril).

The Pearson correlation test indicates that there is a significant relationship between the concentration of the secretagogue and the relative amount of diacetyl αMSH, where a low concentration (10^{-11} M) evoked a larger relative amount of diacetyl αMSH than a high (10^{-7} M) concentration ($P<0.05$). There was no correlation between the type of secretagogue used and the relative amount of the isoforms.

*Table 1- relative amounts of αMSH isoforms in superfusates. For each hypothalamic messenger, only superfusate fractions obtained with the highest and lowest concentration secretagogue were analysed. To enable detection of the αMSH in the samples, preference was given to superfusate fractions with a high concentration of αMSH. The relative amounts of αMSH in the samples were expressed as percentages of the total amount (values ± SEM, N=4). Significant differences between these values are expressed as *=P<0.05; **=P<0.01 and ***=P<0.001. There was a significant inverse correlation (P<0.05) between concentration (10^{-7} or 10^{-11} M) and the relative amount of di-acetylated αMSH.*

Secreta-gogue	Concentra-tion	%desac αMSH	%monoac αMSH	%diac αMSH	Significance
Basal release	0	37.5 ± 5.6	44.8 ± 4.5	17.7 ± 2.6	mono vs. di **
CRH	10^{-7} M	32.2 ± 8.7	53.7 ± 8.5	14.2 ± 3.7	mono vs. di *
CRH	10^{-11} M	34.4 ± 1.9	40.3 ± 1.6	25.3 ± 3.0	mono vs. di **
TRH	10^{-7} M	28.8 ± 2.1	47.9 ± 2.7	23.4 ± 1.1	des vs. mono **
					mono vs. di ***
TRH	10^{-11} M	28.8 ± 4.3	47.5 ± 5.3	23.7 ± 1.3	mono vs. di *
DA	10^{-7} M	33.5 ± 5.7	50.7 ± 7.0	15.9 ± 3.1	mono vs. di *
DA	10^{-11} M	21.2 ± 6.2	54.8 ± 4.8	24.0 ± 5.6	des vs. mono *
					mono vs. di *
MCH	10^{-7} M	31.2 ± 1.1	45.5 ± 9.0	23.3 ± 2.6	Non significant
MCH	10^{-11} M	25.6 ± 3.0	48.6 ± 1.9	25.8 ± 4.8	des vs. mono **
					mono vs. di **

Discussion

Hypothalamic regulators of αMSH release in red porgy

CRH

The release of αMSH from the pituitary gland of red porgy was stimulated by high concentrations of corticotropin releasing hormone (CRH). In vertebrates (Danger et al., 1989; Roubos, 1997) including fish (Lamers et al., 1994; Rottlant et al., 2001), release of αMSH is modulated by a multitude of hypothalamic messengers. CRH is the main hypothalamic messenger that stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary pars distalis which promotes the secretion of cortisol by the interrenal tissue. This hypothalamic-pituitary-interrenal axis is activated in the response to an acute stressor (Mommsen et al., 1999; Wendelaar Bonga, 1997). CRH-stimulated release of αMSH as we now report for red porgy has also been found earlier, for example for tilapia (Lamers et al., 1994) and gilthead sea bream (Rottlant et al., 2001). Very similar EC_{50} values for CRH were reported for tilapia (10^{-8} M) and gilthead sea bream (1.2×10^{-8} M). When comparing the concentration-response curve we present here and the one obtained by Rottlant et al. (2001), it appears that for gilthead sea bream a stimulation of αMSH release was obtained only by concentrations as high as 10^{-7} M, which was the same for red porgy in our study. Red porgy and gilthead sea bream are phylogenetically closely related and both belong to the Sparidae family. This may account for the similarity and relative insensitivity observed.

TRH

In red porgy, the release of αMSH was stimulated by thyrotropin-releasing hormone (TRH) in a concentration-dependent fashion. The EC_{50} for TRH of 7.4×10^{-10} M is compatible with a physiological function of TRH in this fish. TRH has also been reported to stimulate the release of αMSH in amphibians (Roubos, 1997) and thus this effect may be more wide-spread in vertebrates. Indeed, in various other fish species (Lamers et al., 1991; Rottlant et al., 2000; Tran et al., 1989) TRH appears to stimulate MSH release. However, in tilapia stimulation of αMSH release from isolated pars intermedia is characterised by an EC_{50} of 1.6×10^{-7} M (Lamers et al., 1994), which is considerably higher than the value found here for red porgy. This indicates that the sensitivity of tilapia pars intermedia for TRH is lower than the sensitivity of red porgy pars intermedia, but such differences can also derive from differences in experimental set up and design. Rottlant et al. (2000) compared the response of pituitary glands to CRH and TRH in control and crowded gilthead seabreams. A concentration of 5×10^{-8} M TRH caused a twelve-fold absolute increase in αMSH release. Unfortunately, in this study the concentration of CRH tested was 10^{-9} M, which differed from the TRH concentration and therefore hampers comparison between the two secretagogues. We tested the same concentration range for both TRH and CRH in

our study, and the present results show that CRH only stimulated αMSH release at 10^{-7} M, in contrast to TRH, which stimulated over a wider range of concentrations. For most hormones, plasma concentrations (physiological levels) are in the picomolar to nanomolar range (Green et al., 1991; Lamers et al., 1994). For TRH, we found stimulation of the release at concentrations up from 10^{-10} M. The EC_{50} of the dose-response curve for TRH was 7.4 $x10^{-10}$ M, while the EC_{50} for CRH stimulated αMSH release was at least 10^{-8} M (a value derived tentatively from Figure 1A). These findings indicate that in red porgy pituitary glands, the melanotrope cells are more sensitive to TRH than to CRH. Interestingly, an (absolutely spoken) much higher release of αMSH was achieved by CRH stimulation. With these findings in mind we suggest that basal release of αMSH in cultured red porgy is regulated by TRH, whereas CRH may represent a signal to evoke surges of corticotropic hormone(s). Lamers et al. (1994) suggested a stress paradigm in tilapia, in which TRH-stimulated αMSH release may be involved in chronic stress adaptation, while the response to an acute stressor usually involves stimulation of ACTH release by CRH (Wendelaar Bonga, 1997). If cultured red porgy indeed experience (aquaculture-related) chronic stress, the high αMSH levels that arise from this response may also promote skin darkening.

DA

DA has an inhibitory action on the release of αMSH consistent with a D2-receptor-mediated action. DA is a neurotransmitter involved in behavioural responses, amongst others. DA-ergic drugs influence the behaviour in a variety of teleostean fishes (feeding behaviour in weakly electric fish, electric behaviour, aggressive behaviour; Mok et al., 1998). In cichlids, DA increases locomotor activity via a D1 receptor in telencephalic circuits. This locomotor activity may serve to find the most suitable or safe habitat. Mok et al. (1998) suggest that DA-ergic neurons that mediate responses to fearful stimuli may therefore be activated during stress. DA has also been reported to have a direct effect on the release of αMSH (Hagan et al., 1996; Lindley et al., 1990; Omeljanuk et al., 1989). We demonstrate here that in red porgy, DA inhibits the release of αMSH. For a number of fish and vertebrate species, DA (Danger et al., 1989; Lamers et al., 1991; Omeljanuk et al., 1989) or DA agonists (Hagan et al., 1996) were shown to inhibit the release of αMSH in a concentration-dependent fashion. Lindley et al. (1990) reported that during stress in rats, the inhibitory tone of DA decreases and, by doing so, enables a higher release of αMSH in response to a stressor. This situation may also occur in fish, but little is known about the activity of DA-neurons during stress and the inhibitory control of DA on αMSH release in (stressed) fish. DA-ergic systems may be involved in the stress response in at least two ways, namely by influencing behaviour and via direct modulation of the stress axis.

MCH

We show that MCH directly inhibits the release of αMSH by the pituitary gland at picomolar concentrations and this is in line with the reported picomolar concentrations of this hormone in blood plasma of fish (Green et al., 1991). The release of αMSH was inhibited at all concentrations tested. Indeed, MCH has previously been identified as an important colour changing agent through its aggregating effect on chromatophores (e.g. Burton et al., 2000; Fujii, 2000; Nery et al., 1997; Oshima et al., 2001) but it is also recognized as a factor that influences the hypothalamus-pituitary-interrenal (HPI-) axis in teleost fish (Barber et al., 1987; Green and Baker., 1991; Wendelaar Bonga, 1997). In trout, MCH inhibits ACTH release both *in vivo* and *in vitro* (Baker, 1994). As yet indirect evidence was provided for effects of MCH on cortisol levels during stress modified by background adaptation, where white backgrounds caused higher MCH levels combined with lower cortisol levels (Green et al., 1991). In tilapia, it was shown that concentrations of MCH below 10 µM inhibit αMSH release from the pituitary gland (Gröneveld et al., 1995). In red porgy plasma levels of MCH are not known, but in untreated trout the plasma MCH concentration is in the picomolar range (Green et al., 1991), indicating that under normal conditions in these fish MCH probably has a very small inhibitory effect on the αMSH release. The clear effect of MCH in rainbow trout could not be demonstrated in other fish species, yet it is generally assumed that *in vivo*, MCH has an inhibitory effect on the release of αMSH from the pituitary, which is confirmed here in an *in vitro* set up.

αMSH acetylation modulating agents

In red porgy, the release of αMSH from the pituitary gland can be stimulated by TRH and CRH, and inhibited by MCH and DA. Analysis of the composition of the αMSH released in the superfusate showed that, independent of the secretagogue used, monoacetyl αMSH is the dominant form, with des- and diacetyl αMSH being present in smaller and similar amounts respectively. On the other hand, irrespective whether a stimulatory or inhibitory secretagogue was tested, we found that a higher percentage of di-acetylated αMSH (compared to mono- and des-acetylated MSH) was released at low secretagogue concentrations (10^{-11} M) than at the highest concentrations tested (10^{-7} M), and we take this observation as evidence for differential release. Kishida et al. (1988) revealed that in the trout pituitary gland desacetyl αMSH is the dominant form. However, this is not a common finding as in the carp pituitary gland diacetyl αMSH is the most dominant form. In tilapia and in gilthead sea bream, monoacetyl αMSH is the dominant form in the pituitary gland (Arends et al., 2000; Lamers et al., 1991). Red porgy is phylogenetically most related to gilthead sea bream (Sparidae), which may be an explanation for the fact that we also found monoacetyl αMSH as the dominant form released by the pituitary gland.

Little is known about the bioactivity of the different isoforms in red porgy or in the other species mentioned above. However, the acetylation state of a peptide can be essential for its bioactivity (Keller et al., 1994; Mountjoy et al., 2003). A study on the bioactivities and half-lives of the three αMSH types in rabbit (Rudman et al., 1983) indicated that a higher acetylation state could slow down the degradation of the peptide. In salmon, mono-acetylated αMSH was more active than desacetyl MSH in stimulating melanophore dispersion in a frog test (Kawauchi et al., 1984). In our study, the total amount of MSH released at 10^{-11}M of a stimulatory secretagogue was much lower than at 10^{-7} M. If a more bioactive isoform is released in higher amounts under these conditions, it would compensate for the reduced total amount. For an inhibitory secretagogue, the total release of MSH is lower at 10^{-7} M than at 10^{-11} M. If this αMSH is also composed of relatively less bioactive isoforms, the inhibitory effect would be enhanced.

For red porgy, testing the three isoforms for their potency to disperse melanophores will provide clues on the bioactivity of these peptides at the peripheral level, thereby also exploring the relative impact of the isoforms on the overall skin pigmentation of the fish.

Acknowledgements

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Photographs

Red porgy (*Pagrus pagrus*) captured from the wild.

Red porgy gevangen vanuit het wild.

Cultured red porgy (*Pagrus pagrus*).

Gekweekte ***red porgy.***

Laboratory stock tilapia (*Oreochromis mossambicus*), adapted to a (from left to right:) white, black or glass (grey) background.

Tilapia uit het lab, geadapteerd aan een (vlnr:) witte, zwarte of glazen (grijs) achtergrond.

Chapter 3

Effects of husbandry conditions on the skin colour and stress response of red porgy, *Pagrus pagrus*

Van der Salm, A.L., Martínez, M., Flik, G., Wendelaar Bonga, S.E.

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Abstract

Red porgy, *Pagrus pagrus*, is a potential candidate for aquaculture. However, a marked darkening of the body occurs after capture of wild fish and during farming of cultured animals, reducing the market value. In fish, skin pigmentation is hormonally controlled and the main hormone involved in skin darkening, α-melanophore-stimulating hormone (αMSH), is not only involved in pigmentation but also in the regulation of the response to stressors. In this study, several environmental conditions were evaluated for their potency to influence skin colour and to evoke a stress response. Background colour was the main factor in controlling skin pigmentation. A light background colour restored the lightness value of the skin up to levels found in wild red porgy (L*=~70). The background effect was enhanced by applying blue illumination. Light intensity had no clear effect on the body colour, but a high density of fish had a negative effect on the lightness. Plasma parameters (cortisol, αMSH, glucose, lactate and osmolality) were not influenced by background colour. A stocking density of 25kg/m^3 did not evoke a stress response in contrast to earlier studies on red porgy, nor influenced the body colour. We propose that this difference can be attributed to the number of fish per volume of water, which was lower than in other studies. This indicates that the number of fish per volume of water rather than the density in kg fish per volume of water is the relevant factor. Furthermore, we suggest that the culture of adult red porgy can be optimised by maintenance of fish on a light background, thereby restoring the body colour to a more natural hue, without affecting the stress response.

Introduction

The red porgy, *Pagrus pagrus,* is a fairly new species in aquaculture. Its geographical distribution ranges from the British Isles to Senegal in the Eastern Atlantic and from North Carolina to Argentina in the Western Atlantic. Red porgy is also found in the Mediterranean and Adriatic seas (Kentouri et al., 1995; Mihelakakis et al., 2001; Pajuelo and Lorenzo., 1996). In commercial fisheries, red porgy is highly appreciated for its appearance and meat quality and this, together with a growing concern about overfishing of this species (Vaughan and Prager, 2002), makes it a very suitable candidate for aquaculture. It is a bottom-dwelling fish which lives in sublittoral waters. Juveniles occur in abundance in shallow parts of the ocean (at 20-50 m depth) on sandy bottoms where smaller prey is plentiful; adult fish prefer deeper waters (to 250 m depth) and larger crustacean prey (Labropoulou et al., 1999). Preliminary research shows that red porgy can be successfully bred in captivity (Hernandez-Cruz et al., 1999; Kentouri et al., 1994, 1995). Growth and survival compare well to those of gilthead sea bream (*Sparus auratus*) and sea bass (*Dicentrarchus labrax*), two species already successfully produced in aquaculture (Divanach et al., 1993).

A problem encountered in rearing red porgy is a darkening of the body after capture of wild fish and during farming (Kentouri et al., 1995). The red-silver colour of the body changes into an overall dark grey, most prominently in the tail and fins. This skin darkening severely reduces the attractiveness and market value of the fish.

Colour changes in fish are often related to stress. The main pigmentation controlling hormones α-melanophore stimulating hormone (αMSH) and melanin-concentrating hormone (MCH) are pleiotropic and not only control skin pigmentation but also regulate the response to stressors (Arends et al., 2000; Burton and Vokey, 2000; Green and Baker, 1991; Gröneveld et al., 1995; Lamers et al., 1992). During stress, the hypothalamus-pituitary-interrenal axis is activated (Wendelaar Bonga, 1997). Besides adrenocorticotropic hormone (ACTH), the pituitary gland releases αMSH that induces cortisol release from the interrenal tissue, as has been demonstrated for the tilapia, *Oreochromis mossambicus* (Lamers et al., 1992). Classically, αMSH is considered the main hormone causing dispersion of the melanin granules in melanophores and the subsequent darkening of the skin. MCH has opposite effects and causes pallor (Burton and Vokey, 2000). It is released from the hypothalamus and for a number of fish species it was shown to inhibit αMSH release (*e.g.* Gröneveld et al., 1995; van der Salm et al., Chapter 2). Moreover, MCH exerts a direct effect on cortisol release in fish (Green et al., 1991). Elevated plasma cortisol levels are generally used as the main indicator for stress and activation of the HPI axis in fish (Mommsen et al., 1999; Wendelaar Bonga, 1997). In red porgy, preliminary research on the stress response indicates that resting cortisol levels are generally low and crowding can evoke a mild increase in cortisol levels (<10 ng/ml resting levels and up to 40 ng/ml during crowding; Rotllant et al., 1997; Rotllant and Tort, 1997).

In vertebrates in which the pigmentation of the skin can be changed by hormonal stimulation, the colour of the background and the illumination are determining factors for the intensity and/or the pattern of skin pigmentation (Crook, 1997; Duray et al., 1996; Healey, 1999; Papoutsoglou et al., 2000; Rottlant et al., 2003; Sugimoto, 1993). In addition, temperature may also have an impact on the colour (Fernandez and Bagnara, 1991).

Through analysis of different environmental factors that can have an influence on the skin colour (background colour, illumination intensity and light spectrum) and the effect that aquaculture related stressors (crowding) have on pigmentation, we hope to identify conditions for the cultivation of red porgy that will have a positive effect on the maintenance of the natural skin colour.

Materials and Methods

Experimental set up

Two separate experiments were performed in the spring and autumn of 2003 in the Institute for Marine Biology of Crete (IMBC, Greece). Throughout all experiments, fish were kept in 500L circular polyester tanks filled with natural sea water, continuously replaced with a mixture of fresh seawater and recycled water. Salinity (psu) of the water was 40 and water renewal 100 %/h. Fish were kept under normal day-night rhythm (16L: 8D). Temperature of the water was monitored daily (ranging between 21.6 - 23.3 °C), together with the oxygen levels of the water (4.7 - 5.7 mg/l). Fish were fed with self-feeders containing INVE™ (Dendermonde, Belgium) *Pagrus* feed (crude protein, 50%; crude fat after hydrolysis, 16 %; crude fibre, 2 %; crude ash, 10 %; phosphorus, 1.4 %; vitamin A, 12,500 IU; vitamin D3, 2,500 IU; vitamin E, 300 mg; vitamin C, 2000 mg; copper sulphate + copper, 5 mg; ethoxyquinone; butylated hydroxytoluene).

Experiment 1 - Background colour and light intensity

In the first experiment, 200 fish of around 120 g were obtained from the IMBC hatchery and divided over 8 tanks to study background adaptation to white and red backgrounds under high or low light intensities (1.8 ± 1.0 vs. 0.5 ± 0.1 $\mu mol.m^{-2}.s^{-1}$ respectively (where 1 µmol equals 6.022×10^{17} photons), averaged for different depths and areas within the tanks). This experiment was performed in duplicate tanks randomly located in light-proof enclosures (two tanks per enclosure). During acclimation, fish were kept at a density of 10 kg/m^3 on a black background and under full (visible) spectrum illumination. After an initial sampling point (time 0; 2 fish per tank) the background colour of the tanks was changed and the lamps (Philips, TLD 36W) were replaced by blue spectrum (475 nm) fluorescent aquarium lamps (Marine-Glo, Hagen, Deutschland; Table 1A). In case of the low light intensity treatment, one lamp was fitted while in the high light intensity treatment two lamps were placed. In the latter treatment, the distance between the water surface and the lamps was decreased until the desired light intensity had been reached.

The background colour was effectuated by mounting specially developed nylon bags that fitted precisely into the tanks. To mount the background bags, fish were netted from the tanks and temporarily kept in 50 L grey buckets. A drainage pipe painted in the same colour as the bags was mounted in the middle of the bottom of the tank to allow adjustment of the water volume. The whole procedure took about 15 min per tank.

Table 1- design of experiment 1 (A) and experiment 2 (B). A high light intensity (HLI) corresponds with 1.8 ± 1.0 µmol.m^{-2}.s^{-1} while in low light intensity groups (LLI) we measured 0.5 ± 0.1 µmol.m^{-2}.s^{-1}. Control, low densities (LD) were 10kg/m^3 and crowded, chronically stressful high densities (HD) were 25kg/m^3. Fish were kept on a black (BBG), red (RBG) or white background (WBG).

A

Label	Light Spectrum	Background colour	Light Intensity	Density
BS-RBG-HLI	blue	red	high	low
BS-RBG-LLI	blue	red	low	low
BS-WBG-HLI	blue	white	high	low
BS-WBG-LLI	blue	white	low	low

B

Label	Light Spectrum	Background colour	Light Intensity	Density
FS-BBG-LD	full	black	low	low
FS-BBG-HD	full	black	low	high
BS-BBG-LD	blue	black	low	low
BS-BBG-HD	blue	black	low	high
FS-RBG-LD	full	red	low	low
FS-RBG-HD	full	red	low	high
BS-RBG-LD	blue	red	low	low
BS-RBG-HD	blue	red	low	high
FS-WBG-LD	full	white	low	low
FS-WBG-HD	full	white	low	high
BS-WBG-LD	blue	white	low	low
BS-WBG-HD	blue	white	low	high

After a sampling session, the level of the water was lowered to maintain equal densities of fish. To this end, the drainage pipes were replaced with shorter ones. Five fish per tank were sampled at 0, 2, 8, 16 and 30 days.

Experiment 2 – Crowding stress, background adaptation and illumination

In the second experiment, 120 fish of around 380 g were used (generously supplied by Interfish S.A., Halanari, Greece). We studied the effects of white, red

and black backgrounds, compared two densities of fish (control density of 10 vs. crowded density of 25 kg/m^3; achieved by placing 10 fish in 500 L of water vs. 10 fish in 160 L of water, respectively) and we applied two light spectra: blue spectrum and full spectrum light (light intensities approximately 0.5 µmol.m^{-2}.s^{-1}). Upon arrival at the institute 10 fish per tank were divided over 12 experimental tanks (Table 1B). From that moment on, fish were fed with self feeders containing INVE™ Pagrus feed. Fish were sampled at day 8, 16 and 30 after transfer to experimental tanks.

Sampling

Immediately following capture, skin colour parameters of the fish were determined (see below); next the fish were euthanized in 0.2 % phenoxyethanol. Blood was drawn from the caudal vessels (typically 1 - 1.5 ml), with syringes containing 5 µl of 2 % Na-EDTA to prevent clotting, and 50 µl (= 0.5 TIU) of aprotinin to prevent proteolysis. The blood was spun at 4°C for 5 min at 1500 rpm, after which the supernatant plasma was stored in Eppendorf vials and quickly frozen.

Colour analyses

To determine the pigmentation response of red porgy we used a tristimulus colorimeter (Hunter Lab MiniScan™ XE, Hunter Lab, Reston, USA). We used the CIELab colour space concept (see Figure 1). According to this method, the X, Y and Z values for coloration that are measured by the colorimeter are translated into a lightness value (L*, ranging between 0 for black and 100 for white), a colour value from red (a*) to green (-a*) and a colour value between yellow (b*) and blue (-b*; Trujillo et al., 1996). These values were transformed into specific chromatic attributes, namely the observable colour (e.g. red, blue, yellow) named hue (h*), and the colour saturation (or brightness) which is named chroma (C*). The equations for these transformations are:

$$h^* = \arctan(b^*/a^*)$$

$$C^* = (a^{*2} + b^{*2})^{0.5}$$

Colour measurements were usually taken immediately after capture to prevent darkening of the skin as a result of capture. Skin colour was assessed just behind the head in the dorsal part of the body and in the body area in front of the tail, at the left side of the fish.

Physiological parameters

The αMSH concentration in the plasma was determined as described by Arends et al. (1999). The antiserum used for the αMSH radio immunoassay cross-reacts for 100% with des-, mono- and diacetyl αMSH (Vaudry et al., 1978), and was used in a final dilution of 1:60,000. Previous work on red porgy samples (van der Salm, Chapter 2) showed that this RIA is suitable for determination of red porgy αMSH in plasma and culture medium. Immunocomplexes were precipitated by 7.5 % (w/v) polyethylene glycol and 2.5 % (w/v) bovine serum

albumin (van Zoest et al., 1989). The detection limit was 25.2 pg/ml of sample. To determine cortisol concentrations, a RIA was used as described in detail by Arends et al. (1998). Radioactivity was quantified using a Cobra II γ-counter (Packard Instruments).

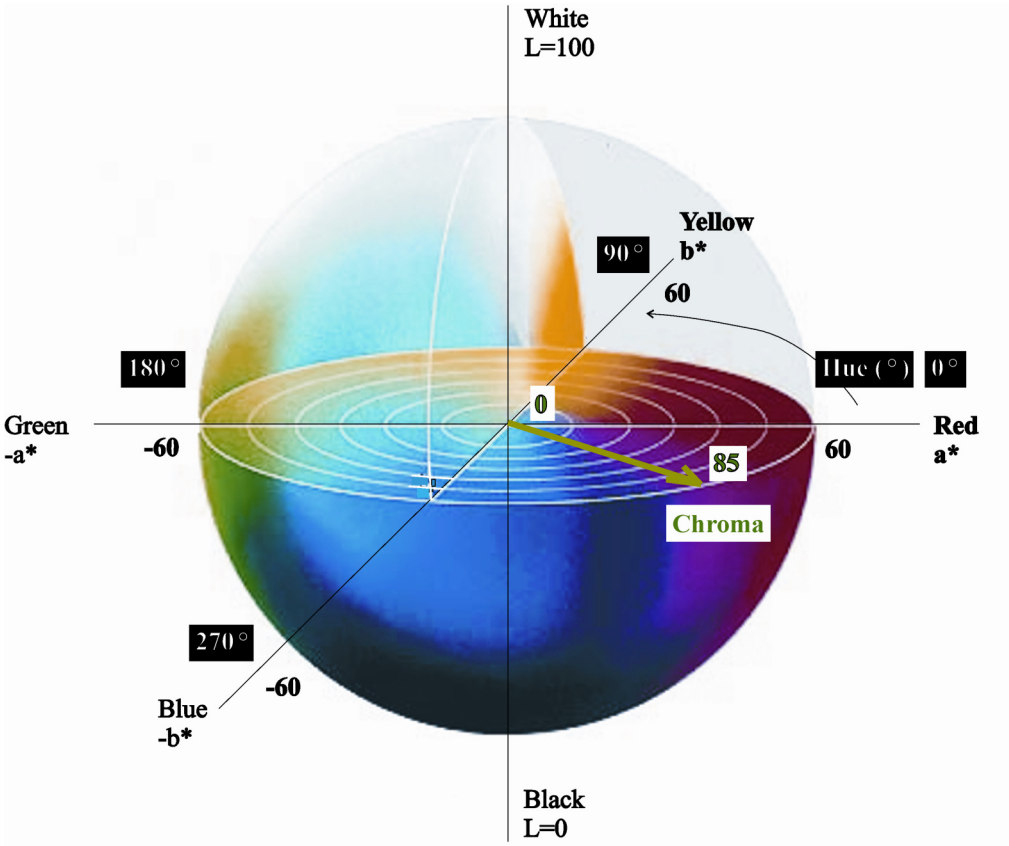

Figure 1- a 3D representation of the CIELab colour space model used in this study. Colour can be described by three factors: the actual colour nuance (hue; 0°=pure red, 90°=pure yellow, 180°=pure green and 270°=pure blue), the brightness of the colour (chroma; the brighter the colour, the higher the chroma) and the darkness or lightness of the colour (the L-value; black=0 and white=100). The a* (value between 60 = red and –60 = green) and b* (value between 60 = yellow and –60 = blue) are the ground values for calculating hue and chroma. For example, pure white has an L*-value of 100 and a chroma of 0.*

Plasma glucose and lactate concentrations were determined with commercial kits from Sigma or with a Stat Profile® pHOx® Plus L Analyser (Nova Biomedical, Waltham, USA). Blood osmolality was measured with an

Osmomat 030 (Gonotec, Berlin, Germany). Na^+, Cl^- and K^+ of plasma were measured by pHOx®.

Statistics

Parameters were compared between groups using two-way analysis of variance (ANOVA) for the first experiment and a three-way ANOVA for the second; followed by Bonferroni or Dunnett C post-hoc tests to assess significance between mean values when the ANOVA indicated significant differences. Correlations between parameters and environmental factors were assessed using non-parametric Spearman's Rho correlation testing (all tests performed with SPSS 11.5 statistical software). The colour parameter hue, being an angle, was transformed and analysed for statistical differences following circular statistics as described by Zar (1999). Differences between groups were assessed by the Watson-Williams test. Statistical differences were accepted at $P<0.05$. Values are shown as means ± standard error of the mean (SEM).

Results

Colour

In the first experiment, the L*-value had increased already 2 days after exposure of fish to a white background (Figure 2A). The L*-value for fish kept on white backgrounds (WBG) was significantly higher than that for fish on a red background (RBG) throughout the experiment. This was in line with a decrease in the number of scale melanophores in WBG fish, while melanophore cell size and melanin dispersion were not influenced by background. There was no noticeable effect of light intensity. Chroma was higher in red background fish, a difference that was significant from D2 onwards (Figure 3A). Hue was not significantly affected by the different background colours or by the light intensity, and ranged between 43° and 69°.

In the second experiment, the L*-value was again highest in the fish kept on WBG (Figure 2B; the correlation between L*-value and increasing lightness of the background was statistically significant with $P<0.01$; $r^2 = 0.21$). In WBG fish kept in blue spectrum (BS), the values were higher than in fish kept in full spectrum (FS) light. For the fish on RBG or on a black background (BBG), the L*-value was not significantly different between BS or FS, nor between low density or high density (LD or HD). In the fish used for the second experiment, the L*-value was generally lower. The hue ranged between 49° and 62° and was not significantly affected by any of the environmental parameters, although values were generally lower in HD groups. The chroma (C*) was higher in dark background fish (RBG and BBG; Figure 3B). C* could be increased by HLI combined with BS. In crowded, HD fish, C* was lower compared to control, LD fish; in FS the C* was higher than in BS.

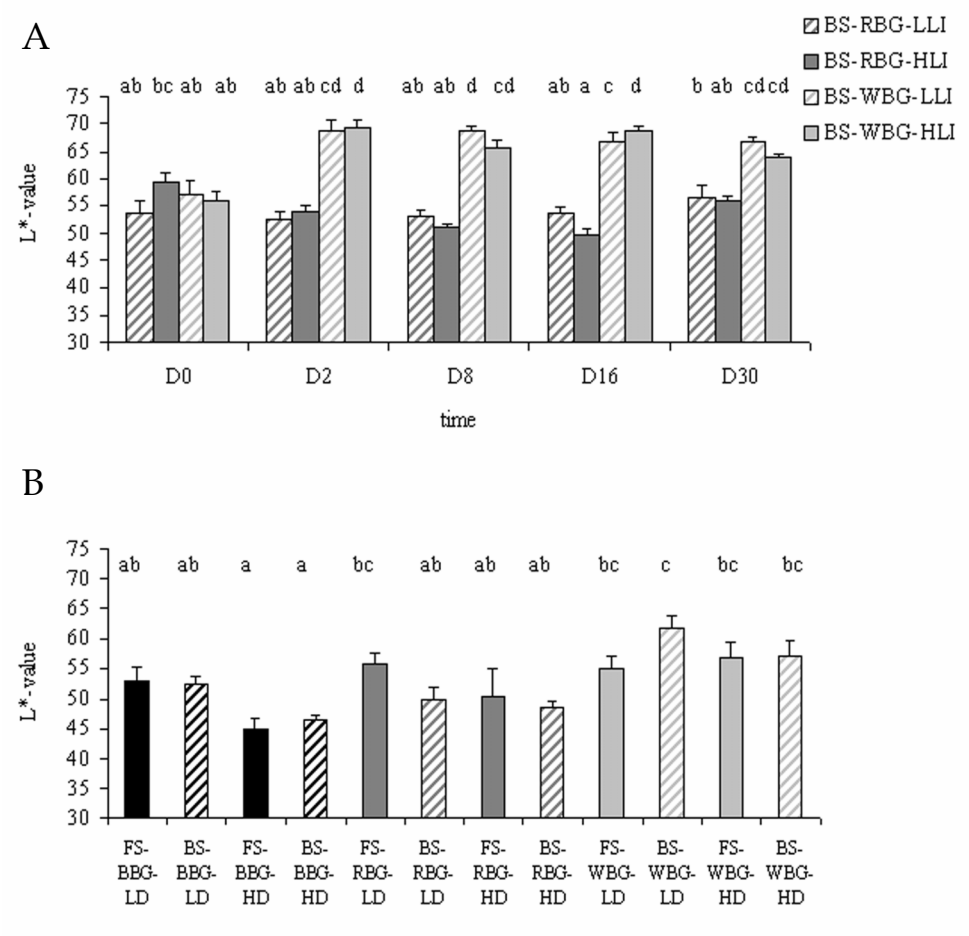

Figure 2- A) The L-values for fish exposed to a white (WBG) or red (RBG) background under high (HLI) or low (LLI) light intensity. L*-values are higher in fish kept on WBG, B) the L*-values for fish exposed to a black (BBG), red (RBG) or white (WBG) background, under full (FS) or blue (BS) spectrum in two different densities: control, low densities (LD, 10kg/m^3) and crowded, chronically stressful high densities (HD, 25kg/m^3), for 30 days. Again L*-values are highest in WBG fish. The other factors show no influence. Statistical differences between values are indicated by different letters (ab, bc or cd: P<0.05; ac or bd: P<0.01 and ad: P<0.001).*

Physiology

Upon a change of background in the first experiment, the cortisol values of the plasma increased in all groups at day 2, but returned to basal from day 8 onwards (Table 2). There were no consistent and significant differences between groups.

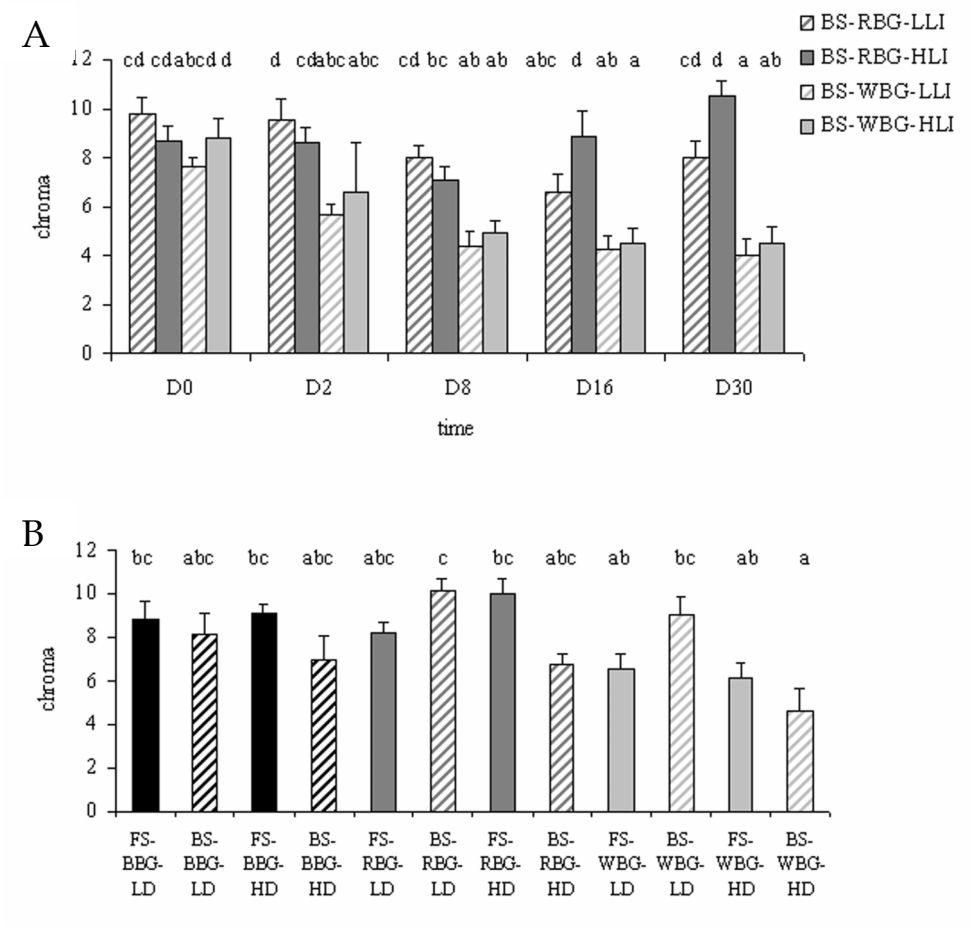

Figure 3- A) The chroma of fish exposed to a red (RBG) or white (WBG) background under high (HLI) or low (LLI) light intensity. The chroma is higher in RBG fish, B) the chroma of fish exposed to a black (BBG), red (RBG) or white (WBG) background, under full (FS) or blue (BS) spectrum in low (LD) and high (HD) densities for 30 days. In WBG fish the chroma is lower than in the fish kept on a dark background. Statistical differences between values are indicated by different letters (ab, bc or cd: P<0.05; ac or bd: P<0.01 and ad: P<0.001).

αMSH concentrations were not influenced by background, yet up to day 16 there was a non significant increase in all groups. The plasma levels of glucose did not differ significantly between groups, while lactate levels were generally higher in HLI groups. The osmolality of the plasma did not differ between

backgrounds in the first experiment, although at day 2 values in all four treatment groups were significantly lower than at the remaining sampling points.

In the second experiment, cortisol levels showed a high variability and significant differences could not be detected (Table 3). This variation can be ascribed to the handling of the fish required to assess the colour parameters. αMSH levels also exhibited this level of variation between treatments. The glucose levels did not differ between backgrounds, but were generally lower in BS fish. Lactate levels were lower in most HD groups. In the second experiment, we explored the osmolality in more depth than in the first experiment and determined ion levels in addition (Table 3). Na^+ levels were generally lower in BS fish and in HD fish compared to the equivalent FS and LD groups of fish, respectively. K^+ levels were also lower in most HD groups compared to the corresponding LD groups. Cl^- concentrations were not significantly influenced by any of the environmental variables.

Discussion

A light background colour in combination with blue light illumination increases the lightness of the skin of cultured red porgy from an L*-value of around 40 in control fish up to an L*-value of 70. The saturation of the colour, the chroma, is higher in cultured fish kept on darker backgrounds with a maximum of around 10. For wild red porgy, the L*-value is in the range of 60-80, while the chroma is approximately 15 (M. Pavlidis, personal communication). The hue does not differ between wild or cultured fish and is around 57° in both cases (red-orange colour, see Figure 1). A high density has a negative effect on the colour parameters. Therefore, to induce a skin colour in cultured red porgy that is comparable to that found in wild specimens, the fish should be provided with a light coloured background while high rearing densities may best be avoided.

Background colour

Exposure to a black or white background colour has widely been used in the past to study the regulation of pigmentation (Burton, 1993; Fernandez and Bagnara, 1991; Sugimoto, 1993). We focussed on the effect of background colour on the skin pigmentation of the fish as well as on its potential stress-inducing effect. While the size of our experimental fish differed between the two experiments presented, their colouring response was similar. Background colour is the most dominant environmental cue to enhance the pigmentation of red porgy to a more natural colour. Background colour as applied in our study did not evoke a stress response: plasma cortisol, glucose and lactate concentrations were similar, irrespective of background colour. Moreover, the plasma concentration of αMSH did not affect pigmentation of red porgy, as we conclude from the absence of correlations between αMSH levels and L*, chroma or hue.

Table 2- plasma cortisol, αMSH, glucose, lactate and osmolality values in red porgy kept on red (RBG) or white backgrounds (WBG) under high (HLI) or low (LLI) blue illumination (BS; experiment 1). Statistical differences between values are indicated by different letters (ab or bc: experiment 1). Statistical differences between values are indicated by different letters (ab or bc: P<0.05; ac: P<0.01).

Parameter	Group	D0	D2	D8	D16	D32
Cortisol (ng/ml)	BS-RBG-HLI	15.6±6.2	138.0±37.8	40.7±24.0	36.3±17.0	20.8±14.1
	BS-RBG-LLI	2.5±0.6	76.4±42.6	33.0±17.0	22.0±10.8	92.8±19.6
	BS-WBG-HLI	1.1± 0.1	99. 7±35.4	50.6±21.0	59.3±27.0	34.9±15.4
	BS-WBG-LLI	1.0±0.1	125.3±48.4	42.6±16.1	20.4±8.5	45.3±18.1
αMSH (µM)	BS-RBG-HLI	43.2±15.8	40.7±9.1	66.5±12.8	142.5±49.4	36.6±10.5
	BS-RBG-LLI	27.2±5.7	29.3±3.6	49.5±19.1	97.2±25.1	29.9±5.9
	BS-WBG-HLI	27.1±5.0	43.3±10.0	77.0±18.8	54.2±6.3	29.9±4.8
	BS-WBG-LLI	13.6±4.0	45.1±10.0	42.9±8.5	105.1±18.5	20.4±3.7
Glucose (mM)	BS-RBG-HLI	2.92±0.16	2.38±0.16	2.83±0.37	1.90±0.13	1.94±0.16
	BS-RBG-LLI	2.80±0.16	2.39±0.22	2.08±0.15	2.01±0.16	2.26±0.11
	BS-WBG-HLI	3.27±0.48	2.35±0.20	2.44±0.19	1.81±0.07	1.98±0.07
	BS-WBG-LLI	3.32±0.37	2.69±0.32	2.02 ±0.12	2.37±0.13	2.23±0.19
Lactate (mM)	BS-RBG-HLI	1.33±0.20	0.82±0.11	1.35±0.20	0.96±0.12	0.69±0.12
	BS-RBG-LLI	0.55±0.05	0.95±0.10	0.40±0.05 ^{b}	1.05±0.17	0.59±0.10
	BS-WBG-HLI	1.36±0.09 ^{a}	0.87±0.10	0.98±0.11	0.83±0.11	0.60±0.10
	BS-WBG-LLI	0.88±0.16	0.92±0.10	1.00±0.13	1.27±0.37	0.73±0.23
Osmolality (mOsmol)	BS-RBG-HLI	400.5±11.3	345.1±1.5 ^{a}	380.1±3.4 ^{c}	397.6±3.0 ^{c}	377.7±3.5 ^{bc}
	BS-RBG-LLI	379.0±7.4	352.9±4.3 ^{ab}	361.6±2.2 ^{ab}	384±3.1 ^{c}	378.3±4.0 ^{bc}
	BS-WBG-HLI	400.3±15.1	348.5±1.8 ^{a}	390.2±2.4 ^{c}	391.3±3.2 ^{c}	376.8±3.0 ^{bc}
	BS-WBG-LLI	351.7±6.9	355.0±1.9 ^{ab}	383.9±3.7 ^{c}	386.8±7.6 ^{bc}	370.5±1.1 ^{bc}

Table 3- plasma cortisol, αMSH, glucose, lactate and ions (Na^+, K^+, Cl^-) values in red porgy kept on black (BBG), red (RBG) or white backgrounds (WBG) under full (FS) or blue spectrum (BS) illumination in low (LD) or high fish densities (HD) for 30 days. Statistical differences between values are indicated by different letters (ab: P<0.05).

Group	Cortisol (ng/ml)	αMSH (μM)	Glucose (mM)	Lactate (mM)	Na^+ (mM)	Cl^- (mM)	K^+ (mM)
FS-BBG-LD	36.6±34.3	76.7±15.0	3.14±0.15	1.70±0.20	189.8±1.1	173.8±2.9	5.15±0.16
FS-BBG-HD	70.0±8.1	42.9±10.9	4.20±0.35	1.98±0.26	193.2±1.6	170.8±1.6	6.11±0.45
BS-BBG-LD	12.8±8.5	103.3±33.3	4.07±0.33	1.84±0.12	188±1.7	167.6±1.1	6.64±0.60
BS-BBG-HD	268.4±122.7 [b]	81.5±9.7	3.58±0.36	1.15±0.18	184±0.5 [a]	165.3±0.7	5.28±0.50
FS-RBG-LD	12.5±5.9	77.1±16.9	3.84±0.34	2.15±0.55	192.8±2.8	176.8±1.5	5.51±0.32
FS-RBG-HD	153.2±84.2	54. 5±7.0	3.24±0.31	1.87±0.15	195.3±0.5 [b]	172.7±1.4	5.34±0.25
BS-RBG-LD	151.1±59.5	82.9±17.6	3.16±0.22	1.8±0.17	193±0.9	172.4±1.1	5.20±0.24
BS-RBG-HD	49.2±21.1	36.6±6.1	3.07±0.20	1.33±0.15	185.7±1.1	168.3±1.5	5.78±0.18
FS-WBG-LD	183.2±37.5	38.3±8.5	3.07±0.21	1.5±0.16	193±0.8	176.9±2.5	5.55±0.50
FS-WBG-HD	30.2±13.9	50.4±6.1	3.96±0.27	1.17±0.15	190.8±2.0	174±2.8	5.12±0.17
BS-WBG-LD	22.1±7.33 [a]	66.9±8.2	3.02±0.15	1.84±0.08	190.6±1.2	167.5±1.8	6.00±0.26
BS-WBG-HD	123.3±43.3	53.6±5.4	2.95±0.17	2.18±0.32	188±2.7	168.8±1.2	5.54±0.23

In trout, plasma levels of αMSH are higher on a black background than on a white background (Rodrigues and Sumpter, 1984). In the same species, MCH levels are higher in white background adapted fish (Green et al., 1991). In addition to their role in regulation of skin colour, both hormones influence the cortisol release and thus the stress response. MCH inhibits both the release of αMSH (Gröneveld et al., 1995; van der Salm et al., Chapter 2) and of cortisol (Baker et al., 1985). It may very well be that increased levels of MCH reported for a number of fish species adapted to a white background can reduce the level of stress in the animals. Indeed, Papoutsoglou et al. (2000) reported that carp kept on a white background have lower plasma concentrations of cortisol and a higher growth rate, consistent with the energy reallocation that occurs under such conditions (Vijayan et al., 1997). These findings are consistent with our findings in red porgy, and indicate that a white background not only reduces skin darkening but may also enhance the ability of the fish concerned to cope with stress and promote growth. However, this conclusion contradicts the findings of Rotllant et al. (2003) on red porgy. They found that white background adapted fish show an increased release of cortisol *in vitro* in response to ACTH and αMSH. However, the plasma levels of cortisol reported for the fish used in that study did not exceed 40 ng/ml during 23 days of chronic stress, which is indicative of a mild stress response (Wendelaar Bonga, 1997). Moreover, background colour did not alter the *in vivo* response to crowding, similar as we found in the present study. We interpret these findings as indications that background colour does not influence the response to crowding. Therefore, a light coloured background may positively affect the skin colour of red porgy, without evoking a noticeable stress response. We attribute the high variation in physiological parameters observed in some groups to handling stress that was unavoidable with our experimental protocol.

The fast paling response to a white background that we report in this study suggests some form of neural regulation of the melanophores. In 1984, Rodrigues and Sumpter recognized that pigmentation is controlled both hormonally and neuronally. In fish, fast colour changes usually result from neuronal control and longer term morphological colour changes from hormonal control (Fujii, 2000). Generally, fast colour changes involve a quick reallocation (dispersion or aggregation) of pigment granules (melanosomes) within the dermal melanophores, while the morphological colour change involves proliferation or apoptosis of melanophores, which may be combined with increased or decreased sensitivity of the melanophores to regulatory signals (Sugimoto, 1997). A two-day process of paling may be too slow for regulation through nerve fibres directly contacting the skin melanophores. However, the skin colour of red porgy may also be under neuroendocrine regulation of catecholamines. Indeed, plasma catecholamine levels were found to be elevated in fish from a white background (M. Pavlidis, unpublished data).

In both our 30 day studies, we could not establish a relation between plasma αMSH concentrations and skin darkness. A study by Sugimoto (1993)

showed that during adaptation of chemically denervated medaka (*Oryzia latipes*) to a white background, an increased density of melanophores was found. This indicates that in white background adapted fish, neurotransmitters reduce the number of melanophores in addition to their aggregating effect on the melanosomes in these cells (Fujii, 2000). The involvement of neural mechanisms rather than hormonal pathways in the regulation of melanophore size and density could explain the lack of correlation between αMSH concentrations and the darkness of the skin (L*-value).

Light spectrum

In our second experiment we compared the effect of full spectrum illumination, similar as provided by sunlight when fish are kept in sea cages near the water surface, with the effect of a blue spectrum. This was the spectrum seen at 200m depth, where adult red porgy are found (Labropoulou et al., 1999). In fish kept in a blue spectrum, the body shows a lighter colour; the glucose concentration and the osmolality of the blood were lower than in the corresponding full spectrum treated groups. There were no differences in the other parameters.

These findings indicate that blue light may be less stressful for the fish than full spectrum illumination and that it can enhance the effect of a light background. Our results are in line with a study by Volpato and Barreto (2001) who showed that blue light prevents the confinement-induced cortisol peak in Nile tilapia under full spectrum light. In a few other studies on this subject it was concluded that the spectrum did not influence the stress response (Downing and Kitvak, 2002; Head and Malison, 2000). Previous research by Szisch et al. (2004) on red porgy has shown that a blue light spectrum can induce paling of the skin colour in this species.

Light intensity

In the first experiment with fish kept under blue spectrum light at two different backgrounds, we compared the effect of high light intensity with low light intensity and found that a high light intensity further enhanced the effect evoked by background colour.

Studies on the influence of light spectrum and light intensity have focussed mainly on their effect on hatching fish (Boeuf and Le Bail, 1999; Cuvier-Péres et al., 2001; Downing et al., 2002; Fermin and Seronay, 1997). The growth rate of young fish can be stimulated by appropriate light-dark regimes. When the light intensity is too high, this may cause stress and mortality (Boeuf and Le Bail, 1999). However, a high light intensity can also attract prey (Fermin and Seronay, 1997) and especially for visual predators a high light intensity enhances visibility of the prey (James and Heck, 1994). For red porgy in our experimental set up with self-feeders, prey visibility is not important. We found no evidence for a stronger response to stressors in high light intensity fish compared to fish kept in low light intensity and have therefore not included this factor in our second experiment.

Density

A high density had a darkening effect on the skin colour; the L-value was lower in high density fish and both the chroma and hue were also lower in high density than in low density fish. However, in fish kept at high densities, we found lower values for lactate, Na^+ and K^+ compared to the values in fish kept at low densities. Plasma cortisol and αMSH levels did not show a clear relation with density.

In general, crowding results in elevated concentrations of plasma cortisol (Rotllant et al., 1997, 2000, 2003; Tort et al., 1996). In some of the experiments, such an increase of cortisol was transient (Tort et al., 1996), but in general this elevation in cortisol levels of crowded fish persisted throughout the experiments. In our set up, crowding did not result in an increase of cortisol levels. The plasma glucose concentration, a parameter widely used to indicate a stress response (Wendelaar Bonga, 1997) did not vary between high or low density groups in our set up. In red porgy subjected to crowding, glucose levels are either unchanged or slightly elevated (Rotllant et al., 1997, 2000). The lack of response in the red porgy used in our experiments could indicate that the intensity of the stressor applied was low. However, the density applied by Rotllant et al. (1997, 2000, 2003) varied between 7 and 10 kg/m^3 for controls and between 20 to 30 kg/m^3 during crowding. Our experimental design of fish kept at 10 kg/m^3 versus 25 kg/m^3 was similar; however, we used fish of a much larger size than the animals used by Rotllant and co-workers. Where our control tanks and our tanks for applying crowding stress had the same amount of fish but a different volume of water, Rotllant and co-workers have doubled the amount of fish in the same volume of water to evoke crowding stress. Also, with an average body weight of 380 g, less fish are needed to reach a certain density (expressed as kg per volume of water) than when 120 g fish are used. Perhaps, the results obtained by Rotllant and co-workers differ from the results presented here because the number of fish per volume of water is a more important factor for the impact of crowding than the density in kg per volume of water.

Next to that, a study by Pottinger et al. (1995) is one of the few studies to examine effects of stressors on rainbow trout of different age. After applying a one hour confinement stress, they showed that sexually immature fish responded stronger to the stressor than mature fish. Therefore, a smaller size implicating a younger age may be an important factor in the magnitude of the stress response in fish and this could explain why the fish used by Rotllant and co-workers responded stronger to the stressors applied than the fish we have used in the present experiments.

We found as a general trend that the concentration of lactate, Na^+ and K^+ in the plasma of red porgy was higher in low density fish, although there were no significant differences between specific groups. Therefore, these findings again support our hypothesis that a density of 25 kg/m^3 is not stressful to large red porgy (>380 g). However, densities reached in aquaculture far succeeding this level may be stressful, and for smaller, immature fish (120 g) it has been shown

that prolonged exposure to a density of 20 kg/m^3 or higher is indeed experienced by the fish as chronic stress (Rotllant et al., 1997, 2000, 2003; Tort et al., 1996).

We conclude that restoration of the natural red skin colour of red porgy in aquaculture requires an environment with a light background, preferably under light or blue spectrum. The reddish colour may be further enhanced by the application of food with a high concentration of natural carotenoids.

A high density (25 kg/m^3) was not experienced as stressful in our experiments but it had a negative (darkening) effect on the skin colour. While we found no stress inducing effects of these high densities on adult red porgy (380 g), previous research has shown that for immature fish (<120 g), high densities can indeed cause stress. Therefore, we suggest that the densities to rear immature red porgy should be kept low, around 10 kg/m^3.

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Chapter 4

The acute stress response of red porgy, *Pagrus pagrus*, kept on a white or red background

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Abstract

The skin colour of red porgy, *Pagrus pagrus*, can be modified by exposure to different background colours. Red and white background colours brighten the dark skin colour that develops under common culture conditions in red porgy. To assess whether skin colour is also modified by aquaculture related handling stress, we subjected red porgy to 5 minutes of netting stress combined with air exposure. Fish kept on a white background have: 1) a lighter skin colour, which is not influenced by an acute stressor, 2) a less saturated red colour, which significantly decreases 24h post-handling, and 3) a similar hue as fish kept on a red background. The first plasma parameters to rise after application of the stressor are cortisol, αMSH, lactate and Na^+; then, glucose levels increased. Other plasma ions (Ca^{2+}, Cl^- and K^+) were not affected up to 2 h post-stressor, but had decreased at 8 h and 24 h after handling. Plasma pH decreased over the first 2 h post-handling, indicative of plasma acidosis upon air exposure. The acidosis then coincided with increases in plasma lactate and αMSH levels. The rise in cortisol preceded that in αMSH levels, from which we conclude that following acute stress in red porgy, plasma cortisol release is controlled by ACTH, perhaps in combination with a sympathic stimulation.

Introduction

In fish, exposure to harmful or potentially harmful stimuli causes an integrated stress response similar to that in mammals. The chromaffin cells in the head kidney receive signals from the hypothalamus via the sympathetic nervous system to produce and release catecholamines. The hypothalamus further releases neuroendocrine factors that can induce the release of corticotrope signals from the pituitary gland that stimulate the interrenal steroid producing cells of the head kidney to produce and release cortisol into the bloodstream. These two pathways are called hypothalamic-sympathetic-chromaffin cell (HSC) axis and hypothalamic-pituitary-interrenal (HPI) axis, respectively (Wendelaar Bonga, 1997).

In aquaculture, fish are frequently exposed to stressful situations such as handling and confinement. For red porgy, *Pagrus pagrus*, a relatively new species in aquaculture belonging to the Sparidae (Kentouri et al., 1995; Pavlidis et al., 2000), it was demonstrated that stress evoked by crowding results in moderately elevated cortisol levels (Rottlant et al., 1997; Rottlant and Tort, 1997). During handling-induced stress these values doubled. In a study by Arends and co-workers (1999), the stress response of the gilthead sea bream (*Sparus auratus*), another sparid fish, was tested by exposure of the fish to handling. The response of fish that had previously been exposed to crowding stress was significantly lower than fish that had not been previously stressed. Such attenuation of the acute stress response during chronic stress has been described in detail (Wendelaar Bonga, 1997).

An important problem in commercial culture of red porgy is a marked darkening of the body. Wild red porgy exhibit a pink, silvery body coloration, but in aquaculture the body colour darkens and the brightness decreases markedly. A hormone classically related to skin darkening is α-melanophore-stimulating hormone (αMSH). This hormone induces skin darkening by causing the stellar-shaped pigment cells (melanophores) to disperse their black pigment (melanin) granules within the cytoplasmic processes of the cell (Bagnara and Hadley, 1973; Baker et al., 1984). More recently, it has been shown that αMSH is also involved in the stress response in some fish species (Baker et al., 1984; Lamers et al., 1992; Wendelaar Bonga, 1997), and in one report αMSH was designated as a corticotrope hormone (Balm et al., 1995).

Adaptation of fish to a dark background can raise the blood levels of αMSH (Baker et al., 1984; Burton, 1993), although this phenomenon appears to be species-specific. In red porgy, previous research by our group (van der Salm et al., Chapter 3) and others (Rotllant et al., 2003) on background adaptation has shown that the skin colour lightens on a white background. Also under blue light illumination red porgy develop a lighter body colour (Szisch et al., 2004).

The darkening of the body colour of red porgy in aquaculture suggests that this fish experiences aquaculture conditions as a stressor. The purpose of this study was to investigate the stress response and the pigmentation response of red

porgy to handling (netting combined with air exposure). We further studied whether the background colour had a modulating effect on these responses, by keeping the fish on a red or a white background.

Materials and methods

Experimental setup

In October and November 2002, 100 fish weighing 372 ± 59 g were generously supplied by Interfish S.A. (Halanari, Greece). Upon arrival in the Institute of Aquaculture (Heraklion, Crete), fish were transferred to 500 L circular polyester tanks filled with natural sea water, continuously replaced with a mixture of fresh and recycled seawater. Salinity of the water was 40 psu and water renewal 100 %/h. Fish were kept under normal day-night rhythm (16L:8D) and under a blue light spectrum (475nm; Marine-Glo, Hagen, Deutschland, 40W) in light proof enclosures of two tanks per enclosure. One lamp per tank was placed 130 cm above the water surface, resulting in a light intensity of 0.5 ± 0.1 $\mu mol.m^{-2}.s^{-1}$.

Temperature of the water (ranging between 21.6 - 23.3 °C) was monitored daily, together with the oxygen levels of the water (these varied between 4.7 - 5.7 mg/l). Fish were fed with self-feeders containing INVE™ (Dendermonde, Belgium) *Pagrus* feed (crude protein, 50 %; crude fat after hydrolysis, 16 %; crude fibre, 2 %; crude ash, 10 %; phosphorus, 1.4 %; vitamin A, 12,500 IU; vitamin D3, 2,500 IU; vitamin E, 300 mg; vitamin C, 2000 mg; copper sulphate + copper, 5 mg; ethoxyquinone; butylated hydroxytoluene).

Two weeks prior to the start of the experiment, fish were divided over 10 experimental tanks (10 fish per tank) and allowed to adapt to tanks with white (WBG fish) or red (RBG fish) coloured walls, mounted as described by Van der Salm et al. (Chapter 3). The experiment consisted of exposure of fish to 5 min of netting while being held above the water surface; in this way confinement and air exposure were simultaneously applied. Fish were sampled immediately after netting (time 0.05 h), or 2.00 h, 8.00 h and 24.00 h after netting. Non-stressed fish, kept under similar background and illumination conditions, were sampled as controls one week prior to the netting experiment.

Sampling

Immediately following capture, some colour parameters of the skin of the fish were determined with a portable spectrophotometer (Hunter Lab Miniscan™ XE) as described by Van der Salm et al. (Chapter 3). The parameters brightness (L*), observable colour or hue (h*) and colour intensity or chroma (C*) were calculated according to the CIELab system (Trujillo et al., 1996). Colour samples were taken from the control group, and from the experimental fish at the t = 0.05 h sampling and after 24 h. Next, the fish were euthanized in 0.2 % phenoxyethanol. At all sampling points, blood was drawn from the caudal vessels, with syringes containing 35 µl of 2 % Na-EDTA to prevent clotting, and

50 µl (= 0.5 TIU) of aprotinin to prevent proteolysis of the peptide hormones. The blood was spun at 4 °C for 5 minutes at 1500 rpm, after which the supernatant plasma was stored in Eppendorf vials and quickly frozen.

Physiological parameters

The αMSH concentration in the plasma was determined as described by Arends et al. (1999). The antiserum used for the αMSH radio immunoassay cross-reacts for 100% with des-, mono- and diacetyl αMSH (Vaudry et al., 1978), and was used in a final dilution of 1:60,000. Immunocomplexes were precipitated by 7.5 % (w/v) polyethylene glycol and 2.5 % (w/v) bovine serum albumin (van Zoest et al., 1989). The detection limit was 25.2 pg/ml of sample. To determine cortisol concentrations, a RIA was used as described in detail by Arends et al. (1998). Radioactivity was quantified using a Cobra II γ-counter (Packard Instruments, Boston, USA). Plasma glucose, lactate, ions (Na^+, Cl^-, K^+ and Ca^{2+}) and pH were measured with a Stat Profile® pHOx® Plus L Analyser (Nova Biomedical, Waltham, USA).

Statistics

Parameters were compared between groups using two-way analysis of variance (ANOVA), followed by Dunnett C post-hoc tests to assess significance between mean values (all tests performed with SPSS 11.5 statistical software, SPSS Inc., Chicago, USA). Data on hue, an angular variable, were transformed and analysed according to circular statistical methods described by Zar (1999). Statistical differences were accepted at $P<0.05$. Values are shown as means ± standard deviation (SD).

Results

Colour parameters

The L*-value was consistently higher in WBG fish compared to RBG fish (Figure 1A). This difference was significant at all time points ($P<0.05$). L*-values were not influenced by the stressor, since there were no significant differences through time for fish kept on either background. The mean chroma (C*) was higher in RBG fish at all time points (Figure 1B). At 24.00 h post-stressor, C* of WBG had decreased almost by 50% ($P<0.01$) compared to the C* value immediately post-netting. C* values of RBG fish were not influenced by the stressor. The hue of the fish was significantly elevated in RBG fish immediately after application of the stressor (0.05 h) compared to the control and 24.00 h RBG values (Figure 1C; $P<0.05$). Hues of fish kept on a white background were not significantly altered. On average, the hue was around 50° (red-orange) for fish kept on both backgrounds.

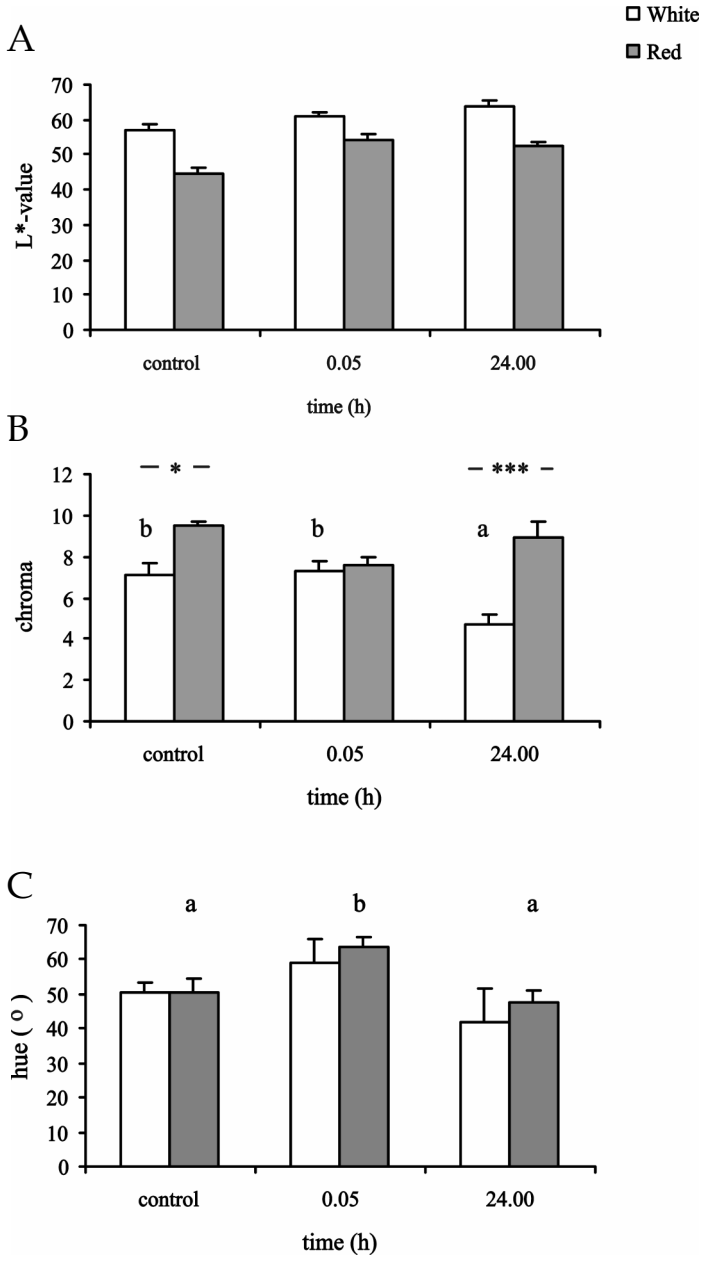

*Figure 1– the lightness (A), saturation or chroma (B) and the visible colour hue (C) of red porgy skin adapted to a red and white background prior to (control), immediately after (0.05 h) and 24 h after (24.00) 5 min of netting and air exposure. Significance between background colour treatments is indicated by * = P<0.05; *** = P<0.001. Significant differences between time points are indicated by different letters (black for white background fish and grey for red background fish; a vs. b: P<0.05).*

Physiological parameters

Na^+ levels in the plasma showed an increase immediately after application of the stressor (Figure 2A; P<0.05). Levels increased up to 2.00 h post-netting (significantly higher than control values for WBG fish) and had decreased to control values at 8.00 and 24.00 h. The strongest rise immediately after application of the stressor was seen for plasma lactate and cortisol levels (Figures 2 and 3). Plasma lactate levels were significantly elevated at this time point (0.05 h) in WBG fish (Figure 2B; P<0.05). At 2.00 h after netting, levels were significantly higher than control values in fish from both backgrounds (P<0.01). At 8.00 and 24.00 h, levels had decreased below control levels in both groups. The pH of the plasma showed a significant decline at 2.00 h post-netting compared to control values in WBG fish (Figure 2B). In RBG fish, plasma pH did not change significantly over time.

Plasma glucose did not increase until 2.00 h post-netting (Figure 2C); in RBG fish this increase was significant (P<0.05). At 24.00 h, plasma glucose values had decreased significantly compared with the 2.00 and 8.00 h levels (P<0.05).

Plasma cortisol values remained elevated compared to control values up to 8.00 and 24.00 h after netting (Figure 3A). The increase was significant at 2.00 h post-netting (both backgrounds; P<0.01), where levels had increased up to 300 ng/ml. For RBG fish, plasma cortisol levels remained significantly elevated above control levels at all time points. Plasma αMSH levels showed a similar response pattern over time (Figure 3B).

Plasma Ca^{2+} levels tended to be elevated at 2.00 h post-netting in both groups, but these were not significantly higher than those in controls (Table 1). Ca^{2+} values decrease below control values at 8.00 and 24.00 h, but again this difference was not statistically significant. Plasma K^+ and Cl^- levels remained similar to control values at 0.05 h and 2.00 h post-netting, and showed a significant decrease at 8.00 and 24.00 h compared to the levels at 0.05 and 2.00 h post-netting (Table 1).

Table 1 – Plasma ion levels through time in red porgy adapted to a red and white background after 5 min of netting and air exposure (0.05 h). Significance between values is denoted by: a vs. b = P<0.05. Values sharing the same letter are not significantly different; nsd = no significant differences between any of the time points.

	K^+		Cl^-		Ca^{2+}	
	Red	***White***	***Red***	***White***	***Red***^{nsd}	***White***
Control	5.53±0.18	5.53±0.23^b	180.7±2.6	179.3±1.7^b	1.18±0.04	1.14±0.03
0.05 h	6.02±0.26^b	5.62±0.37	185.4±2.8^b	191.2±2.7^b	1.14±0.06	1.18±0.07
2.00 h	6.28±0.74	5.62±0.13^b	179.4±4.7^b	187.1±2.8^b	1.23±0.11	1.38±0.05^b
8.00 h	3.86±0.12^a	4.06±0.15^a	165.3±1.5^a	164.0±1.1^a	0.99±0.04	1.03±0.06^a
24.00 h	3.86±0.12^a	3.93±0.24^a	162.5±1.7^a	166.2±4.4^a	1.10±0.05	1.12±0.08

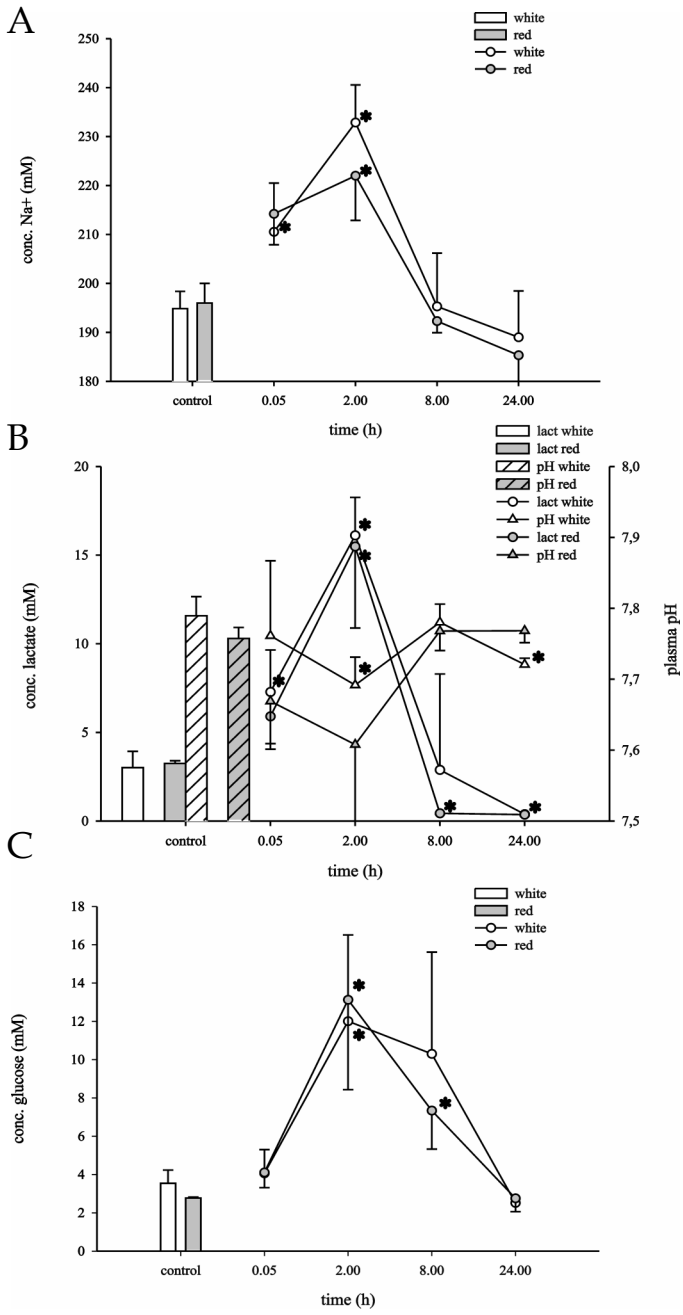

*Figure 2– the plasma concentrations of Na^+ (A), lactate and pH (B) and glucose (C) through time in red porgy adapted to a red and white background after 5 min of netting stress and air exposure. Significant differences compared to control values are indicated by * = P<0.05 or ** = P<0.01. There were no significant differences between background colour treatments at any time point.*

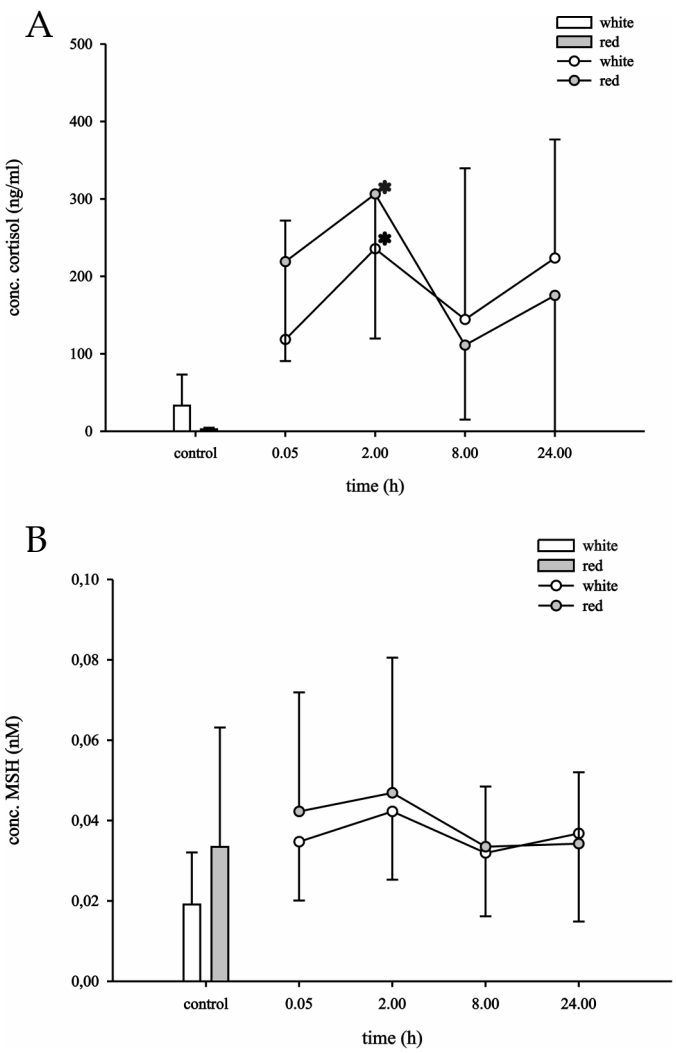

*Figure 3– plasma levels of cortisol (A) and αMSH (B) through time in red porgy adapted to a red and white background after 5 min of netting and air exposure. Significant differences compared to control values are indicated by * = P<0.05. There were no significant differences between background colour treatments at any time point.*

Discussion

Whereas background adaptation clearly modifies the skin colour of red porgy, it does not alter the response to an acute stressor (netting the fish above the water surface). The physiological response to the stressor does not differ between RBG and WBG fish, and shows marked changes in plasma Na^+, lactate, cortisol and αMSH concentrations. These αMSH levels are not correlated with skin colour in red porgy.

After three weeks of background adaptation, red porgy shows differences in colour pattern that are mainly attributable to the lightness of the skin. On a white background, fish generally have a lighter skin as indicated by a higher L*-value, whereas the chroma and hue do not differ significantly between groups. However, chroma was higher in RBG fish than in WBG fish at all time points. Upon stressing, the L* value does not change compared to control values. Chroma does not change after application of the stressor, yet was decreased in WBG fish after 24h. Apparently, fish became paler (also indicated by increased L* value through time in the WBG fish). Transient colour changes in fish are a well known phenomenon due to motile responses of the chromatophores (Burton, 2002; Fujii, 2000), but colour changes after stress are not well documented. Studies on social interactions in flounder indicate that social stress can result in colour pattern changes to signal submission or dominance (Höglund et al., 2000, 2002). Too little is known about social interactions in red porgy to translate these findings to a possible function of stress-induced paling. The hue of the body increased immediately after applying the stressor only in the RBG fish, although a similar trend of change was observed in WBG fish. This indicates a transient shift of red porgy body colour from a red to a more yellow colour upon being stressed. However, since this shift comprises only a few degrees, the colour change is minor and unlikely to be of physiological significance.

The lack of correlation between background colour and αMSH levels indicates that αMSH may not be involved in the skin pigmentation of red porgy. In previous studies on red porgy and related species such as the gilthead seabream, *Sparus auratus*, similar results were obtained. Szisch et al. (2004) and van der Salm et al. (Chapter 3) found that adaptation to different backgrounds and illumination did not influence the plasma αMSH levels of red porgy. In amphibians the involvement of αMSH in background adaptation has been reported on numerous occasions (Fernandez and Bagnara, 1991; Hogben and Slome, 1931; Roubos, 1997), but in fish the picture is less clear. Eel, trout and catfish have been reported to show increased plasma levels of αMSH when kept on a black background (Baker et al., 1984), yet in flounder and sea bream such correlations are absent (Szisch et al., 2004). It seems that in the latter species, as in red porgy, other neurohormones such as catecholamines or melanin concentrating hormone (MCH) have a more dominant role in colour change than αMSH.

The stress response of teleost fish comprises three subsequent stages: the primary stress response, characterised by increases in plasma catecholamines and cortisol, the secondary stress response wherein the former hormones influence energy mobilization and disturb hydromineral balance, and the tertiary stress response which mainly involves long term inhibition of growth, immune functions and an inability to cope with additional stressors (Wendelaar Bonga, 1997). In this paper, we show that in red porgy, the primary stress response, after confinement and air exposure during netting above the water surface, is characterised by increased levels of cortisol and αMSH, and lactate and Na^+. The very rapid increases in plasma lactate and Na^+ levels suggest that these rises result from a catecholaminergic stimulation rather than from activation of the HPI-axis, and this corresponds with previous reports (Wendelaar Bonga, 1997). Air exposure leads to hypoxia and plasma acidosis (Arends et al., 1999; Vijayan et al., 1997) and indeed red porgy responds with a decrease in plasma pH at 5 min and 2 h after netting.

While Na^+ levels show an increase immediately after application of netting stress, the levels of all other plasma ions measured are not significantly altered within 2 h after netting. This may indicate a selective gain of ions that enter the fish from the hyperionic environment. After 2 h however, all ion levels return to or drop below basal control levels, suggesting that the permeability of the gills has returned to normal and indicating that the hydromineral balance has actively and quickly been restored.

Upon exposure to an acute stressor such as netting above the water surface, cortisol levels in the plasma of red porgy increase around ten-fold compared to basal cortisol values (0-32 ng/ml). Consensus exists that such swift increases are mediated by a release of corticotropin-releasing hormone (CRH) from the hypothalamus that stimulates the release of adreno-corticotropic hormone (ACTH) from the pituitary gland which, in turn, stimulates cortisol release by the interrenal tissue in the head kidneys (Wendelaar Bonga, 1997). A possible role for αMSH in the release of cortisol in tilapia has been described by Balm et al. (1995). Studies by Lamers et al. (1992) on this species indicate that the CRH-ACTH-cortisol axis predominates during acute stress situations while a TRH-αMSH-cortisol axis can be activated during chronic stress. In this study, the rise in cortisol precedes the rise in αMSH indicating that in red porgy, an acute stressor activates the CRH-ACTH-cortisol axis. Nevertheless, αMSH levels increase during the first two hours after the application of a stressor, irrespective of the background colour. Arends et al. (1999) also showed that 3 min of air exposure led to an increase of αMSH levels in the plasma of gilthead sea bream. These authors suggest that a stressor such as air exposure leads to acidosis in the plasma, resulting in increased release of αMSH. The release of cortisol was not accompanied by an increase of ACTH levels in the plasma, which led these authors to conclude that perhaps the increase of cortisol resulted from sympathic activity: acetylcholine, the neurotransmitter of parasympathic fibres stimulates interrenal cells directly to release cortisol (Arends et al., 1999). With respect to an

involvement of αMSH in cortisol release, it is noteworthy to mention that recent research in carp has shown a direct projection of CRH-containing neurons on the pars intermedia of the pituitary gland (Huising et al., 2004) and thus hypothalamic CRH-neuron activity would result in αMSH release. However, as yet only a melanocortin (MC)-2 receptor has been found in the head kidney of fish, which is selective for ACTH. An MC5 receptor could not be demonstrated and this would exclude αMSH as a corticotrope (Klovins et al., 2004; Metz and Flik, submitted). On the other hand, the pleiotropic nature of this hormone, combined with numerous observations on plasma increases during acute stress indicates that αMSH may have an as yet unknown function in the stress response (*e.g.* food intake or immunological responses; Cerda-Reverter et al., 2003; Luger et al., 2003).

The results of this study suggest that for red porgy, handling induced stress can evoke swift and strong responses, particularly in plasma cortisol, αMSH, lactate and Na^+ levels. Since handling is unavoidable in aquaculture, cultured red porgy will experience stress frequently. However, the observed darkening in red porgy under these conditions can not be attributed to the elevated αMSH levels. Skin colour was not correlated with plasma αMSH levels. Adaptation to a white background could lighten the skin, yet without any involvement of αMSH. Background colour did not alter the stress response to netting; for fish from either a red or white background the response followed a similar pattern: immediate increases in cortisol, αMSH, lactate and Na^+, followed by increased glucose levels and a drop in plasma pH. From 8 h onwards, most parameters were restored.

Our results emphasize the need to evaluate neural and endocrine involvement in skin colour regulation. The current trend to diversify fish aquaculture has brought several species at the attention of researchers. Amongst other factors, this has markedly increased the number of fish species currently studied in the field of body colour regulation, and it is becoming clear that enormous differences occur in the main pathways to regulate the skin colour. For the red porgy, it seems that not αMSH but catecholaminergic pathways are more important in control of skin colour during aquaculture related stress.

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does not necessarily reflect the views of the Commission and in no way anticipates the Commission's future policy in this area.

Chapter 5

Background adaptation and water acidification affect pigmentation and stress physiology of tilapia, *Oreochromis mossambicus*

Van der Salm, A.L., Spanings, F.A.T., Gresnigt, R., Wendelaar Bonga, S.E., Flik, G.

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Abstract

The ability to adjust skin colour to the background is a common phenomenon in fish. The hormone α-melanophore-stimulating hormone (αMSH) enhances skin darkening. In Mozambique tilapia, *Oreochromis mossambicus* L., αMSH acts as a corticotropic hormone during adaptation to water with a low pH, in addition to its role in skin colouration. In the current study, we investigated the responses of this fish to these two environmental challenges when it is exposed to both simultaneously. The skin darkening of tilapia on a black background and the lightening on grey and white backgrounds is compromised in water with a low pH, indicating that the two vastly different processes both rely on αMSH-regulatory mechanisms. If the water is acidified after 25 days of undisturbed background adaptation, fish showed a transient pigmentation change but recovered after two days and continued the adaptation of their skin darkness to match the background. Black backgrounds are experienced by tilapia as more stressful than grey or white backgrounds both in neutral and in low pH water. A decrease of water pH from 7.8 to 4.5 applied over a two-day period was not experienced as stressful when combined with background adaptation, based on unchanged plasma pH and plasma αMSH and Na^+ levels. However, when water pH was lowered after 25 days of undisturbed background adaptation, αMSH levels increased chronically. In these fish, plasma pH and Na^+ levels had decreased, indicating a reduced capacity to maintain ion-homeostasis, implicating that the fish experience stress. We conclude that simultaneous exposure to these two types of stressor has a lower impact on the physiology of tilapia than subsequent exposure to the stressors.

Introduction

Many poikilothermic animals have the ability to adjust the hue (observable colour; Trujillo et al., 1996) of their skin to the colour of their background by a combination of swift (physiological) and longer term (morphological) pigmentation changes (Bagnara and Hadley, 1973). Pigment cells, or chromatophores, can undergo changes in cell number, size, pigment content, pigment dispersion within the cell and/or cell migration. A change in hue that involves a shift from light to dark or *vice versa* results from alterations in the state of black pigment cells, melanophores. During morphological pigmentation changes, numerical density and pigment content of melanophores can be decreased or increased upon adaptation to a white or black background, respectively. Physiological pigmentation changes comprise a relocation of the melanin granules within the cell, enabling a fast regulatory mechanism. In both mechanisms of pigmentation change, the same hormones are involved (Fujii, 2000).

Alpha-melanophore-stimulating-hormone (αMSH) is classically known for its ability to induce pigment dispersion within pigment cells (Bagnara and Hadley, 1973), and is also involved in the regulation of melanin synthesis in mammals and fish (van Eys and Peters, 1981; Halaban, 2000). Next to these pigment regulatory functions, in fish αMSH serves as a satiation signal in feeding behaviour (Cerda-Reverter et al., 2003) and acts as corticotrope in the chronic phase of the stress response (Wendelaar Bonga, 1997). Plasma αMSH levels increase after temperature shock (Sumpter et al., 1985), during confinement combined with air exposure (van der Salm et al., Chapter 4) and during chronic exposure to acidified water (Lamers et al., 1991).

In addition to adaptation to a background, changes in hue can also be part of communication signals (Hulscher-Emeis, 1992; Lamers et al., 1991; Moyle and Cech Jr., 2000). In Mozambique tilapia (*Oreochromis mossambicus* L.), as in many other cichlid species, social status is reflected by the skin colour pattern of the fish, with the dominant male showing a much darker hue than submissive conspecifics. The ability of tilapia to alter the pigmentation pattern of their skin both very quickly and over a longer period of time makes them a very suitable model for background adaptation studies (van Eys and Peters, 1981).

Furthermore, during adaptation of tilapia to low water pH, αMSH-cell activity and plasma levels are significantly higher than in fish kept in water of pH 7.4 (Lamers et al., 1992). Other studies by the same authors indicated αMSH to have corticotropic effects by stimulating directly the release of cortisol from the interrenal tissue in the head kidney (Balm et al., 1995; Lamers et al., 1992).

These findings point to roles for αMSH in tilapia in both pigmentation control and in the stress response. To further identify this role of αMSH in these processes, we studied the interaction between background adaptation and the response to chronic low water pH. We assessed physiological responses (plasma hormones, glucose, lactate, Na^+ and K^+) and determined the pigmentation

response by analysis of scales taken from a defined location and assessing the area of the scale covered by melanophores as a parameter for the darkness of the body.

Materials and methods

Animals

Male and female tilapia, weighing 66 ± 17 gram (means ± sd; n=230) were obtained from laboratory stock, kept in large full-glass tanks. Following a time 0 control sampling (12 fish), fish were distributed over 15 experimental tanks containing 50L tap water of pH 7.8. Water temperature was 24° C and fish were kept at a day/night rhythm of 12 L:12 D. Fish were fed daily commercial tilapia food (Tilapia 3.0, Trouw, Putten, The Netherlands).

Experimental set up

The walls of each experimental tank were covered with self-adhesive black (black background; B) or white foil (white background; W). The control tanks (control full-glass, grey background; G) were fitted with light-permeable one-sided see-through foil. In this way, disturbance of the fish by external movements or other stimuli was kept at a minimum and was similar for all groups, and the fish kept on the full-glass, grey underground served as extra controls, comparable to the background situation experienced in the rearing tanks (Table 1).

A first group of fish was transferred from the stock tanks to the experimental tanks and was allowed to adapt to the different backgrounds for 25 days. Experimental tanks were sampled at day 2, 8 and 25. This group is identified as the N (neutral water) treated group. A second group of fish was transferred and in the first two days after the start of the experiment the pH of the water was decreased from 7.8 to 4.5 by addition of H_2SO_4 (Lamers et al., 1994). These fish were left to adapt to both the different background colours and the low water pH for 25 days and tanks were sampled at day 2, 8 and 25. This group of fish is referred to as the pH group. The third group of fish was left to adapt undisturbed in neutral water to the different backgrounds for 25 days, before the pH of the water was lowered gradually over a two-days period (day 25 to 27) from pH 7.8 to pH 4.5 and kept at this pH for another 23 days (day 27 to day 50). These experimental tanks were sampled at day 27 (immediately after the pH drop) and at days 33 and 50. This group is referred to as the NpH (neutral followed by low pH water) treated group (see Table 1).

Throughout the experiment, pH of the water was monitored daily (Radiometer PHM, Copenhagen, Denmark; pH ranging between 7.4 and 7.9 for neutral water and 4.2 and 4.8 for acidified water) and adjusted if required.

Table 1- experimental set-up. Fish were kept on three different backgrounds (translucent glass as a reference control grey background; a black and a white background) and allowed to adapt in either neutral water for 25 days (N), followed by a pH drop (7.8 to 4.5) over a two-days period after which the pH was kept at 4.5 (NpH); or a pH drop and subsequent low pH immediately after the start of the experiment (pH).

	Background:	**Grey**	**Black**	**White**
Water treatment:	Days of treatment:			
Neutral	0-25	NG	NB	NW
Low pH	0-25	pHG	pHB	pHW
Neutral followed by low pH	25-50	NpHG	NpHB	NpHW

Sampling

Upon sampling, all fish were taken from a tank in a single scoop and euthanized in 0.2 % 2-phenoxyethanol. Blood was drawn from the caudal vessels, with syringes containing 35 µl of 2 % Na-EDTA to prevent clotting, and transferred to Eppendorfs containing 1 TIU of aprotinin to prevent proteolysis. The blood was spun at 4 °C for 10 minutes at 13,500 rpm, after which the supernatant plasma was transferred to fresh Eppendorf vials and quickly frozen.

After blood sampling, whole body pictures were taken of each fish. Then a scale was taken from each fish from the third row of scales below the lateral line on the left-hand side of the fish, approximately the sixth scale to the right of the operculum. Scales were fixed in 35% formalin and photographed later under a Leica Stereoscope (MZFLIII, Leica Camera AG, Solms, Germany) with a Leica DC 500 digital camera. Fixation of the scales did not macroscopically affect melanophore features.

The pituitary gland was dissected and immediately frozen until further analysis.

Light microscopic analysis of the scales

The digital photographs of the scales were analysed for the percentage of the scale covered by the melanin of melanophores. This method does not include the dispersion state or the number of individual melanophores but gives an indication of the lightness or darkness of the scale and therefore of the hue of the body. We used Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose, USA) to measure the area (pixels) of the scales covered by epidermis and then measured the area (pixels) covered by melanin using the "magic wand" selection tool. By dividing the two values, a melanophore coverage expressed as a percentage of the scale (epidermis) was obtained.

Physiological parameters

Pituitary glands were homogenized mechanically on ice in 100 µl of 0.1M HCl. After centrifugation (13,600 rpm, 4° C) for removing membranes and cellular debris, the supernatant was used to assess the total amount of αMSH in the pituitary glands. The αMSH concentration in the plasma and in the pituitary gland was determined as described by Arends et al. (1999). The antiserum used for the αMSH radio immunoassay cross-reacts for 100% with des-, mono- and di-acetyl αMSH (Vaudry et al., 1978), and was used in a final dilution of 1:60,000. Immunocomplexes were precipitated by 7.5 % (w/v) polyethylene glycol and 2.5 % (w/v) bovine serum albumin (van Zoest et al., 1989). The detection limit was 25.2 pg/ml sample. To determine cortisol concentrations, a RIA was used as described in detail by Metz et al. (2003). Radioactivity was quantified using a Cobra II γ-counter (Packard Instruments, Boston, USA). Plasma glucose, ions (Na^+, K^+) and pH were measured with the Stat Profile® pHOx® Plus L Analyser (Nova Biomedical, Waltham, USA).

Statistical analysis

Differences between groups were tested for significance with a two-way analysis of variance (ANOVA), followed by Dunnett's C post hoc tests when significant differences were found. The percentage of scale coverage by melanophores was arcsine transformed (Sokal and Rohlf, 1995) and then tested using the same methods. All values are expressed as means ± SD. Statistical analyses were performed using SPSS 12.0.1 statistical software.

Results

Pigmentation

Two days after transfer to the experimental tanks, scales of fish in neutral water (N) were temporarily lighter on all backgrounds compared to the initial control fish sampled at day 0 (Figure 1A). This paling response was statistically significant only for fish from grey (NG) and white (NW) backgrounds. There was no difference in scale pigmentation between fish just after the pH drop during the first two days (pH group) and fish kept in neutral water for that time. While grey (pHG) and black background (pHB) fish subsequently turned darker again, pHW fish continued to turn lighter throughout the experiment. pHG fish showed lower melanophore coverage values than NG fish, a difference that was significant at day 8 ($P<0.05$). On the other hand, pHW fish showed slightly higher melanophore coverage values compared to NW fish (not significant). NG and NB fish had significantly higher coverage values than NW fish ($P<0.05$) and NB fish were visibly darker (Figure 1B). The pH decline after 25 days of undisturbed adaptation evoked a similar response: NpHB fish showed a transient paling response, while NpHW fish on the other hand showed a slight transient darkening of the body. In the 23 days following this pH drop, NpHG and NpHW

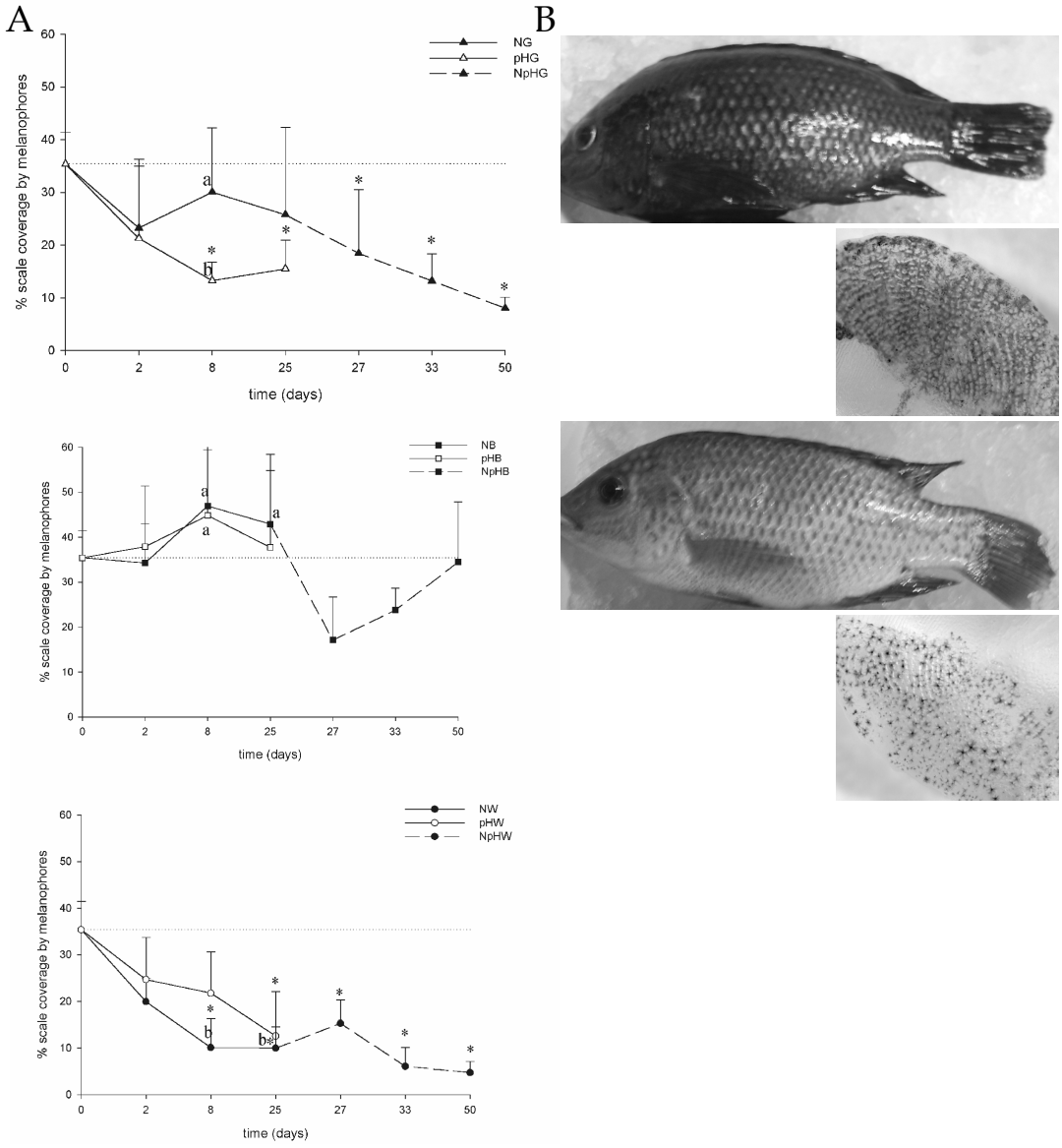

*Figure 1– A) Darkness of the scales as expressed in % of the total area of scale that is covered by melanophores (+ SD). Fish were kept on three different backgrounds (translucent glass resulting in a grey background, ▲; black, ■; and white, ●) undergoing different water acidification treatments (no acidification, N, closed symbols; direct acidifation, pH, open symbols; and acidification after 25 days in neutral water, NpH, closed symbols with dashed line). Significant differences (P<0.05) from time 0 control (indicated by the dotted line in all graphs) are indicated by *; differences between corresponding values from different backgrounds are indicated by letters; a vs. b= P<0.05, B) Tilapia kept in neutral water for 25 days with corresponding scale; upper: black fish, with a scale melanophore coverage of 36.2% and lower: white fish with a scale melanophore coverage of 5.4%*

fish continued to pale further, a process that had started during the first 25 days of the experiment. NpHB fish became darker during this period. The correlation between the darkness of the background and the scale darkness was very significant ($P<0.001$, $r^2 = 0.304$; Table 3).

*Table 2- plasma pH, glucose, lactate and Na^+ values for tilapia kept on a translucent control grey (G), black (B) or white (W) background (±SD). Fish were acidified (water pH from 7.8 to 4.5) either immediately after the start of the experiment (pH group) or first allowed to adapt to the different backgrounds in neutral water (N) before the water was acidified (NpH). The values of these two groups are depicted underneath one another. Values of fish from low pH water are boxed in grey. Significant differences between values are indicated with letters; a vs. b = P<0.05, a vs. c = P<0.01; values significantly different from day 0 initial values are indicated with * (P<0.05).*

day	**pH**		**glucose**		**lactate**		**Na^+**	
	NG; NpHG	**pHG**	**NG; NpHG**	**pHG**	**NG; NpHG**	**pHG**	**NG; NpHG**	**pHG**
0	7.26±0.02		3.5±0.8		1.5±0.1		170±2	
2	7.25±0.09	7.26±0.08	3.6±0.4	3.9±1.0	1.4±0.3^a	1.5±0.4^a	173±3	174±4
8	7.21±0.17	7.34±0.09	4.0±0.4^a	3.9±0.4	1.8±0.6^a	1.4±0.4^a	173±4	170±7^b
25	7.28±0.09	7.22±0.17	3.5±0.2^b	3.8±0.7	1.2±0.4	1.2±0.5	176±6	179±7
27	7.24±0.13		3.6±0.3		1.4±0.5		167±5	
33	7.25±0.06		n.a.		0.8±0.1^{b*}		163±2	
50	7.16±0.22		4.0±0.4		0.8±0.1^{b*}		169±5	
	NB; NpHB	**pHB**	**NB; NpHB**	**pHB**	**NB; NpHB**	**pHB**	**NB; NpHB**	**pHB**
0	7.26±0.02		3.5±0.8		1.5±0.1		170±2	
2	7.30±0.05	7.26±0.05	3.6±0.6	4.0±0.8	1.3±0.2	1.4±0.3	171±4	173±3
8	7.29±0.08	7.28±0.08	3.8±0.9	3.5±0.4	1.1±0.4	1.3±0.4	167±4^b	178±4^a
25	7.30±0.10	7.24±0.13	3.2±0.2	3.3±0.4	1.1±0.3	1.4±0.5	174±6	175±5
27	7.24±0.13		4.0±0.6		1.1±0.1*		174±11	
33	7.27±0.07		3.7±0.2		1.1±0.4		168±3^b	
50	7.17±0.12		3.6±0.3		1.2±0.5		167±5	
	NW; NpHW	**pHW**	**NW; NpHW**	**pHW**	**NW; NpHW**	**pHW**	**NW; NpHW**	**pHW**
0	7.26±0.02		3.5±0.8		1.5±0.1		170±2	
2	7.29±0.06	7.25±0.08	3.4±0.5	5.0±1.8	1.2±0.3	1.4±0.3	172±4^b	175±4^{ab}
8	7.33±0.07	7.26±0.08	3.6±0.4	3.7±0.3	1.6±0.5	1.3±0.3	172±5^{bc}	180±2^{a*}
25	7.23±0.09	7.28±0.09	3.4±0.3	3.6±0.2	1.2±0.4	1.6±0.4	176±6^{ab}	177±6^{ab}
27	7.23±0.10		4.0±0.2		1.1±0.3		165±3^c	
33	7.25±0.09		4.5±0.6		1.5±0.3^a		173±3^b	
50	7.18±0.15		3.8±0.3		1.0±0.3		165±2^c	

Plasma parameters

Plasma pH was not significantly influenced by the gradual acidification of the water in any of the experimental groups (Table 2). Values varied between 7.1 and 7.4 for all fish and showed a trend, on black and white backgrounds, to be lower in the pH fish. Values were generally lower in NpH fish compared to the pH fish, on all backgrounds.

Plasma cortisol levels were not affected by the pH decrease during the first two days after transfer to the experimental tanks (Figure 2A). Only in NG fish a significant increase in cortisol levels was observed (day 2; Figure 2A). For these fish, the levels of cortisol remained higher in neutral water fish until day 25, yet this difference was not significant. pHB and pHW fish showed an increase up to day 8, a rise which was absent in neutral water fish. NpH fish showed an increase in plasma cortisol levels after a two-day decrease in water pH on all backgrounds. This increase was not significant compared to the day 25 values. These fish showed a high individual variation in cortisol values. However, in NpHB fish cortisol levels still increased up to day 33, whereas in NpHW fish a decrease is visible at this time point. These values differed significantly from each other ($P<0.05$).

Plasma αMSH concentrations were not significantly different between fish from different backgrounds throughout the experiment (Figure 2B). Interestingly, αMSH levels did not differ between fish from low pH water and fish from neutral water during the first 25 days of the experiment, but when the pH was lowered after 25 days of undisturbed adaptation in the NpH fish an increase could be seen of αMSH concentrations on all backgrounds, significant in the NpHG fish only. In these fish, αMSH levels declined afterwards, while levels remained elevated in NpHB and NpHW fish.

The total amount of αMSH present in the pituitary glands is shown in Figure 2C. Increases were seen in the first two days after the start of the experiment in both the pH fish and in the NpH fish on all backgrounds, indicating that the pituitary αMSH synthetic machinery was up-regulated. Levels returned to control values after 2 to 8 days. The total αMSH content of the pituitary gland was not affected by background or by water pH, applied either separately or combined.

Plasma glucose concentrations (Table 2) showed no significant differences between backgrounds. Also, water pH did not significantly influence the glucose levels. Lactate levels (Table 2) showed a decline throughout the experiment on all backgrounds, which was not significantly altered by water pH. Plasma Na^+ levels showed an increase during the first 25 days of the experiment in both the low pH and the neutral water fish (Table 2). In pHB and pHW fish higher Na^+ levels were found than in neutral water fish. NpHG and NpHW fish showed a steep drop in plasma Na^+ concentrations. This decrease was significant in NpHW fish. Plasma K^+ levels were not influenced by background or by water pH.

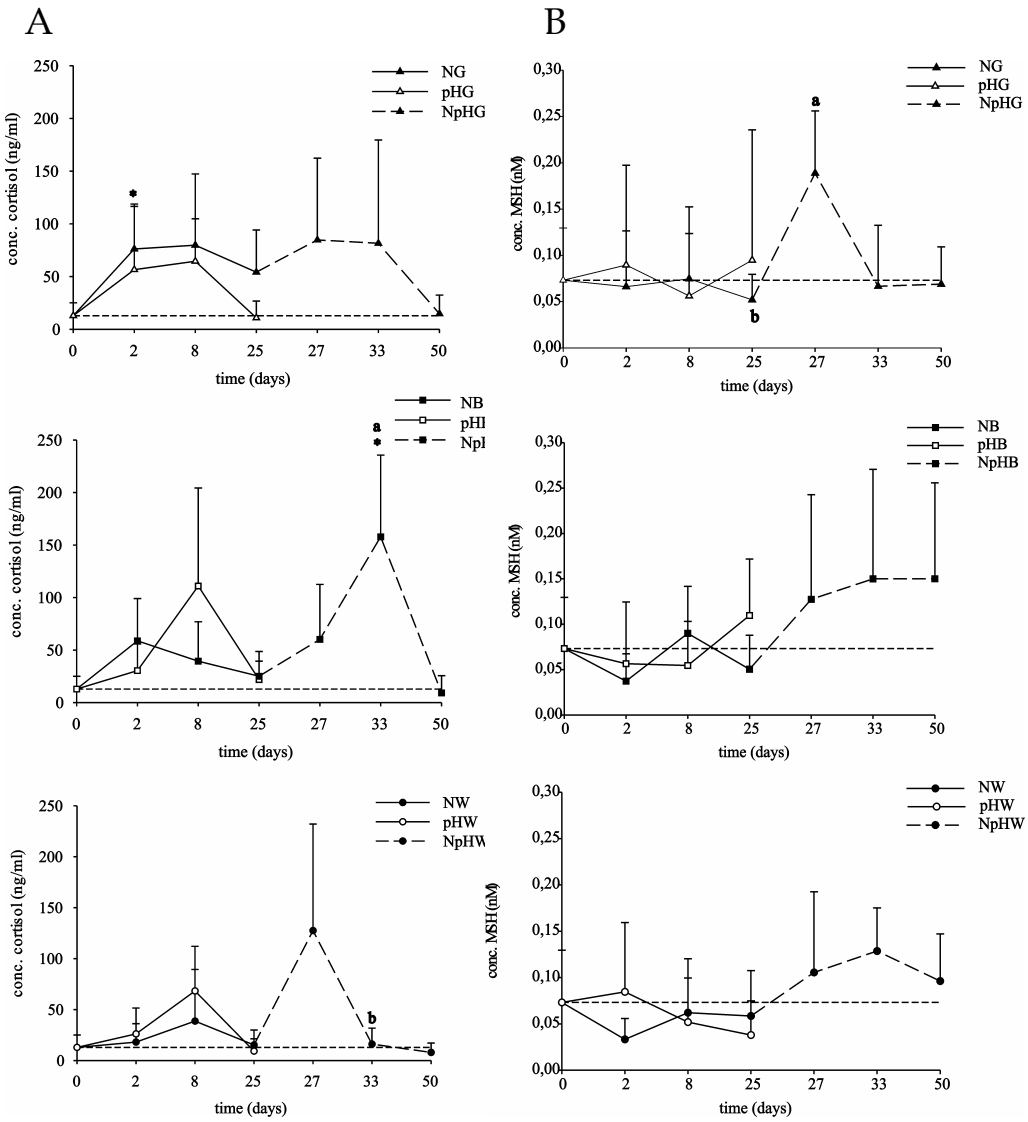

*Figure 2– plasma cortisol (A), αMSH (B) and αMSH content of the pituitary gland (C) for fish kept on three different backgrounds (translucent glass resulting in a grey background, ▲; black, ■; and white, ●) undergoing different water acidification treatments (no acidification, N, closed symbols; direct acidifation, pH, open symbols; and acidification after 25 days in neutral water, NpH, closed symbols with dashed line). Significant differences are indicated by * = different from day 0 control (indicated by dotted line), or different letters; P<0.05 in both cases.*

C

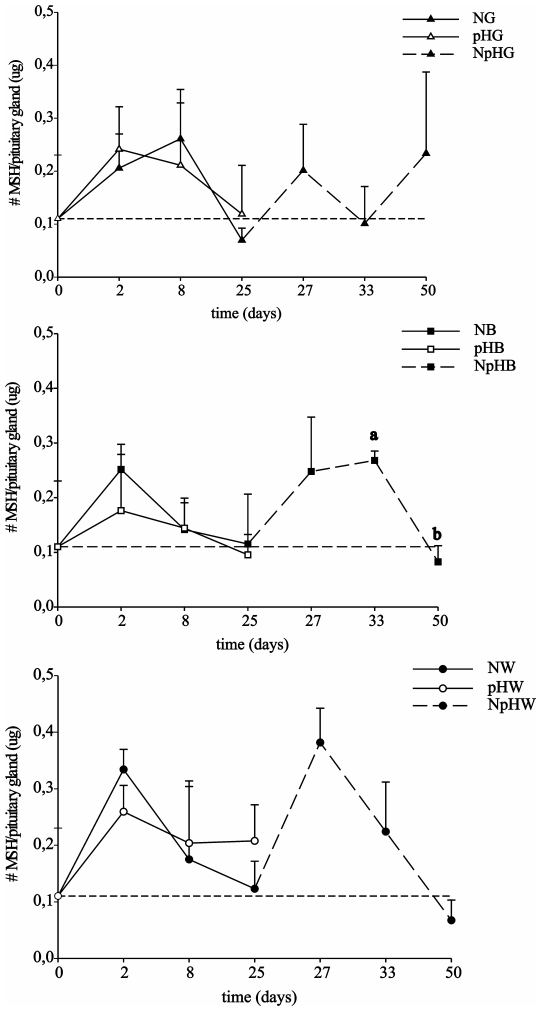

Figure 2, continued

Correlation studies combining all data indicate that overall, plasma αMSH and glucose levels are positively correlated with decreasing water pH, whereas plasma cortisol levels and the scale melanophore coverage values are positively correlated with the darkness of the background (Table 3). Fish with a higher αMSH pituitary content show higher plasma cortisol levels, and lower plasma αMSH concentrations. Plasma Na^+ levels are negatively correlated with low water pH and plasma αMSH levels, but are not affected by plasma cortisol concentrations (Table 3).

*Table 3- correlation table between experimental factors and physiological parameters. A positive correlation is indicated by + and a negative correlation is indicated by -. Significant correlations are indicated by * for a correlation significant with P<0.05 and ** for P<0.01. Non-significant correlations are not shown.*

	pH 7.8 ⇨ 4.5	**B⇨G ⇨W**	**Cortisol**	**αMSH**	**αMSH pituitary**	**Glucose**	**Na^+**	**Scale coverage**
pH 7.8 ⇨ 4.5				+ 0.253**		+ 0.251**	- 0.137*	- 0.309**
B⇨G ⇨W			- 0.152**			+ 0.170*		- 0.551**
Cortisol		- 0.152**			+ 0.444**			
αMSH	+ 0.253**				- 0.228*		- 0.157**	
αMSH pituitary			+ 0.444**	- 0.228*		+ 0.241*		
Glucose	+ 0.251**	+ 0.170*			+ 0.241*			
Na^+	- 0.137*			- 0.157**				
Scale coverage	- 0.309**	- 0.551**						

Discussion

The major conclusions drawn from this study are three-fold. First, a black background appears to be experienced as more stressful than grey or white backgrounds. Second, in acidified water a black background is experienced as an additional stressor (the low water pH itself is a stressor as well). Third, and surprisingly, when the water is acidified following background adaptation the stress response is exaggerated compared to the simultaneous application. Both white and black backgrounds further exaggerate the response to low water pH.

Pigmentation – Effects of background adaptation

Tilapia from a black background were consistently darker than fish from white and grey backgrounds as judged by the scale melanophore coverage values. For the latter groups these scale coverage values were similar. Morphological background adaptation, a long term process, is not so much a result of melanosome reallocation within the cell but rather a result of either degeneration of melanophores on a light background or the formation of new melanophores on a dark background (Sugimoto, 1993, 2000, 2002). The procedure to score the scale coverage by melanophores does not discriminate between scale coverage changes either as a result of changes in melanin dispersion state of individual melanophores, or of changes in cell numbers. However, previous research on Mozambique tilapia has shown that long term background adaptation is indeed a result of changes in the numerical density of melanophores (van Eys and Peters, 1981).

The adaptation to a light background involves degeneration of melanophores. According to Sugimoto et al. (2000) apoptosis of melanophores is induced primarily by sympathetic signals (presumably norepinephrine) to the melanophores. A role was also hypothesised for melanin-concentrating hormone (MCH; Baker, 1993; Sugimoto, 2002), another hormone that affects melanosome motility.

The process of dark background adaptation is under the influence of both peptide hormones (such as αMSH) and catecholamines. The increase in melanophore numbers during dark background adaptation is thought to be under control of αMSH. This hormone can stimulate melanogenesis on the longer term by up-regulation of the expression of melanogenic genes (Sugimoto, 2002). However, the presence of many other factors such as growth factors, keratinocytes, fibroblasts and endothelins, is essential for this process to occur (Sugimoto, 2002). In the present study, plasma αMSH concentrations were not different between fish kept on a grey, a white or a black background, although the darkness of the skin clearly differed between these groups. These results are in line with studies on other fish, in which no correlation was found between the hue of the body and plasma αMSH levels (Baker et al., 1984; Rodrigues and Sumpter, 1984; Zhu and Thomas, 1996). This may relate to changes in isoform frequency: the acetylation degree (αMSH occurs as des-, mono- and di-acetylated

isoforms) is an important determining factor in the bioactivity of the hormone. For instance, di-acetyl αMSH is the most potent corticotrope in tilapia (Lamers et al., 1992).

The involvement of αMSH in pigmentation in fish has been reported for several species, such as catfish, eel and trout (Baker et al., 1984), while in arctic charr, flounder, gilthead sea bream and red porgy such an involvement could not be demonstrated (Arends et al., 2000; Baker et al., 1984; Höglund et al., 2002; van der Salm et al., Chapter 3&4). Earlier conclusions by van Eys and Peters (1981) based on infusion of very high concentrations of exogenous αMSH do not corroborate our findings and we ascribe this to the concentrations used in that study. Probably, infusion demonstrates an effect, but not a real function of αMSH as the concentrations used to reach this effect are non-physiologically high.

Pigmentation – Effects of water acidification

There were no differences in the pigmentation response to the different backgrounds between fish from neutral or low pH water during the pH decline over the first two days of the experiment. However, at the longer term the skin of the low water pH fish was lighter in pHG and pHB fish and slightly darker in pHW fish compared to fish kept at these backgrounds in neutral water. We take this to imply that the low water pH induced a mild stress response that compromised the background adaptation process. On the other hand, acidification after 25 days for two days only temporarily influenced the pigmentation, since from day 27 to day 50 NpH fish restored their former pigmentation pattern and continued their background adaptation process (NpHB fish continued to darken and NpHW and NpHG fish continued to lighten). Apparently, when background adaptation is occurring under low pH water conditions, the background adaptation is slightly hampered. When fish have already adapted for 25 days to a given background, a decrease in water pH no longer influences the pigmentation response. According to van Ginneken et al. (1997) and Lamers et al. (1994), tilapia are able to handle a low pH quite well as long as the water pH is lowered gradually, for instance over a two-day period. The response of the tilapia in our study confirms that a two-day decrease of pH from 7.8 to 4.5 is slow enough not to disrupt hydromineral homeostasis.

Plasma analysis – Effects of background adaptation

Plasma cortisol and glucose showed a significant correlation with background colour. On a black background (NB), cortisol values were generally higher than plasma cortisol levels in NW fish. This indicates that a dark (black) background is experienced as stressful by tilapia. This has also been reported for rainbow trout (Green et al., 1991; Green and Baker, 1991). Black background adapted rainbow trout have higher cortisol levels than white background adapted fish, a difference that was increased by stressors (Green et al., 1991). Interestingly, the increase in plasma cortisol in black background trout was accompanied by decreased MCH levels in the plasma and in several brain regions

(Green et al., 1991). MCH is known to inhibit the release of αMSH from the pituitary gland in tilapia (Gröneveld et al., 1995). For the same species of fish as used in the present study, it was reasoned that the decreased MCH levels and the accompanying loss of the inhibiting tonus on the release of αMSH, may result in increased release of αMSH from the pituitary gland during stress in tilapia, thereby evoking increased release of cortisol (Balm et al., 1995). Our present results are in agreement with this notion.

Plasma analysis – Effects of water acidification

Water acidification, either immediately (pH group) or after 25 days of undisturbed adaptation (NpH group), did not result in significant changes in plasma pH. Plasma pH values between 7.3 and 7.5 have been reported to be normal values in fish (Hirata et al., 2003), which correspond well with the values found in our study. A two-day decrease of water pH was not experienced as stressful in the present study as indicated by the lack of a significant rise in plasma pH and cortisol levels, which is in agreement with previous reports (Ginneken et al., 1997; Lamers et al., 1994), in which either a 6h or a 24h period of gradual water acidification was applied. However, plasma pH levels were generally lower when water acidification (water pH at 4.5) was applied after 25 days of undisturbed adaptation, indicating a reduced capacity to maintain plasma pH levels. Lamers et al. (1994) reported increased activity of melanotrope cells in the pituitary gland of tilapia exposed to a water pH of 4.5 for 7 days. In another study by these authors, plasma αMSH levels were about 5-fold higher in low pH stressed fish (Lamers et al., 1992). In our study, a 3-fold increase was observed only in the NpH fish that had been left to adapt to the different backgrounds undisturbed in neutral water for 25 days before the water was acidified to pH 4.5. For NpHG fish, this increase was only temporary, while NpHB and NpHW fish continued to have elevated plasma αMSH levels.

In the pituitary gland, the αMSH content increased during the first two days of the experiment and during the two-day acidification after 25 days of background adaptation. The pituitary αMSH content did not differ between fish from neutral water and fish from low pH water (both pH and NpH). This indicates that the increased plasma αMSH levels during the final 25 days of the experiment in the NpH fish may have resulted from factors such as increased turnover of αMSH in the pituitary gland or a decrease in αMSH degradation in the plasma. Fish from the NpH group had lower lactate values (significant only in NpHG fish) and lower Na^+ values (significant in NpHW fish) after acidification than fish from the pH groups. Plasma glucose was not affected, whereas cortisol levels increased most in NpH fish. Therefore, acidification starting at day 25, following a 25-day period of undisturbed background adaptation, induces a stronger rise in plasma αMSH and cortisol levels and a stronger decrease in plasma Na+ and lactate levels than acidification starting at day 0, simultaneously with the initiation of the process of background adaptation. This suggests that when fish have been left to adapt undisturbed to a certain background for 25

days, a subsequent acidification of the water has a higher stress-impact on the fish than water acidification immediately after transfer to the experimental tanks. We interpret this as an arousal of the stress axis in these fish.

These findings point to a reduction of the impact of either water acidification or background adaptation when they are both applied simultaneously. Such a reduced stress response to cope with multiple stressors has also been reported in other species of fish and in mammals (Ortuno et al., 2002; Sloman et al., 2002; Wasmund et al., 2002).

We conclude that when tilapia is subjected to different mild stressors simultaneously, this results in a less intense stress response than when exposure to a background or to water acidification is applied separately. This is also supported by our findings that adjustment of the hue to the respective backgrounds under low water pH conditions seems to be compromised compared to background adaptation in neutral water. Interestingly, while the stress response was more intense in the fish that could adapt without any disturbance to the different backgrounds for 25 days prior to the decrease in water pH, these fish showed a recovery of the hue of the skin shortly after the pH decrease and during the remainder of the experiment. This indicates once more that when fish have already accomplished a new homeostatic equilibrium, a subsequent stressor will not interfere with the previous adaptation process. The two adaptation processes studied here apparently do show interference when occurring simultaneously, reducing the individual impact of the stressors applied.

Chapter 6

αMSH, the melanocortin-1 receptor and background adaptation in the Mozambique tilapia, *Oreochromis mossambicus*

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Abstract

The regulation of skin darkness in vertebrates is mediated by α-melanophore-stimulating-hormone (αMSH). For this action, αMSH binds to the melanocortin (MC)-1 receptor, a 7-transmembrane receptor located in melanophore cell membranes. The Mozambique tilapia, *Oreochromis mossambicus*, can change the hue of its skin in response to a change in background, a process that may involve αMSH and the MC1R. Scale melanophores were isolated from tilapia that were acclimated for 25 days to a black or white background and then tested for their sensitivity to des-, mono- and di-acetylated αMSH. On all backgrounds, mono-acetylated αMSH was the dominant isoform present in pituitary homogenates. Mono-acetylated αMSH also had the highest potency to disperse melanosomes. Black background adapted fish showed the highest dispersing response to αMSH, independent of the isoform applied. We determined the nucleotide and amino acid sequence of the tilapia MC1R and show that its expression in skin does not change when animals are acclimated for 25 days to a black or white background, while a clear change in hue is visible. This finding, combined with the absence of differential MC1R gene expression following background acclimation indicates that the increased sensitivity to αMSH is most likely a result of changes in the intracellular signalling system in melanophores of black background adapted fish, rather than up-regulation of the MC1R.

Introduction

Alpha-melanophore-stimulating-hormone (αMSH) plays a role in skin pigmentation of vertebrates (*e.g.* Bagnara and Hadley, 1973). Darkening of the skin and its derivatives (hair, fur, feathers) in mammals and birds is slow and can take up to weeks to fully develop. However, in lower vertebrates such as amphibians and fish, skin melanophores quickly change appearance due to fast movements of dark pigment (melanin) granules, melanosomes, within the melanophore. This enables these animals to show a rapid change of the hue (observable colour of the skin), in response to changes in the background (Healey, 1999; Roubos, 1997). Both the slow darkening process in mammals and birds, and the rapid responses in lower vertebrates can be stimulated by αMSH.

Peptides are often modified posttranslationally by glycosylation, amidation or acetylation. Alpha-MSH is found in three different N-terminally acetylated isoforms: des-, mono- and di-acetylated αMSH. In mammals, as in most other species where post-translational acetylation of αMSH occurs, the major form is diacetyl αMSH (Dores et al., 1993; Keller et al., 1994). In a number of fish species, however, including tilapia, the dominant form is mono-acetyl αMSH (Arends et al., 2000; Dores et al., 1993; Lamers et al., 1991). Acetylation modifies the bioactivity of the peptide (Keller et al., 1994).

The Mozambique tilapia, *Oreochromis mossambicus*, uses body pigmentation as a means to communicate with conspecifics. The social status of an individual is read from its darkness and pigmentation pattern of the skin. Next to that, tilapia is able to adjust the pigmentation of its skin to the background it is kept upon (van Eys and Peters, 1981). A black skin pigmentation can be induced by *in vivo* administration of mono-acetylated αMSH. This indicates that αMSH has melanotropic potency in tilapia and may be involved in the process of background adaptation. Interestingly, during prolonged acid water stress, a shift occurs in the ratio between di- and mono-acetylated αMSH in plasma, in favour of the di-acetylated isoform. This led Lamers et al. (1992) to propose a corticotrope role for di-acetylated αMSH. In a study by Rudman et al. (1983), it was shown that acetylation of the peptide increased the melanotropic potency in a frog skin bioassay (relative potency: di = mono > des-acetylated αMSH) and prevented the degradation of αMSH (di > mono > des-acetylated αMSH). In salmon, mono-acetylated αMSH was also more potent than des-acetylated αMSH to stimulate melanosome dispersion in a frog test (Kawauchi et al., 1984).

The bioactivity of a peptide is determined by the receptor(s) it binds to. In most vertebrates, the control of pigmentation of the skin by αMSH is regulated via the melanocortin (MC) 1-receptor that is localised in the membrane of melanocytes (Cone et al., 1996). This receptor was first designated the αMSH receptor, as binding of αMSH induced a darkening of the skin. In mammals, this MC1R has the highest affinity for αMSH of all five receptor subtypes (named MC1 to MC5 receptors; Schiöth et al., 1995). The MC2R is specific for ACTH and

located mainly in the adrenal tissue. The MC3, MC4 and MC5 receptors can all be found in the brain, the MC3 (*e.g.* adrenal cortex, placenta) and MC5 (e.g. exocrine glands, muscle) receptors are also expressed in a multitude of peripheral organs (Cone et al., 1996). In fish, contradicting findings have been reported. Studies on Japanese pufferfish, *Takifugu rubripes*, and rainbow trout, *Oncorhynchus mykiss*, show that in both species most of the MC receptors have a higher affinity for ACTH than for αMSH (Haitina et al., 2004; Klovins et al., 2004).

In this paper, we compare the scale melanophores of fish adapted to three different backgrounds (black, white and grey background tanks) in their response to the three isoforms of αMSH. We present the cDNA and deduced amino acid sequence of the melanocortin-1 receptor of Mozambique tilapia and have quantitated expression of the MC1R in these background adapted fish.

Materials and Methods

Animals

Male and female tilapia were obtained from laboratory stock (n=24 per background). Fish weighed around 70 g and were kept in 50L tanks containing tap water of pH 7.8. Water temperature was 24° C and fish were kept at a day/night rhythm of 12 L: 12 D. Fish were fed commercial tilapia food (Tilapia 3.0, Trouw, Putten, The Netherlands). The walls of each experimental tank were covered with self-adhesive black (black background; B) or white foil (white background; W). The control tanks (control full-glass, grey background; G) were fitted with light-permeable one-sided see-through foil. In this way, disturbance of the fish by external movements or other stimuli was kept at a minimum and was similar for all groups, and the fish kept on the full-glass, grey underground served as extra controls, comparable to the background situation experienced in the rearing tanks.

Sampling

For scale melanophore studies, three fish from every background were lightly anaesthetised in 0.1% (w/v) 2-phenoxyethanol (Sigma-Aldrich, St. Louis, USA). Scales were removed from the left-hand side of the fish and placed in a physiological salt solution (169 mM NaCl; 5.4 mM KCl; 1.8 mM $CaCl_2$; 1.3 mM $MgCl_2$; 5 mM Tris and 5.6 mM D-glucose) during transfer to the microscope. Fish were returned to the experimental tanks and not sampled again for a week to enable regrowth of scales.

For MC1R expression studies, six fish from every background were euthanized in 0.2% (w/v) 2-phenoxyethanol. Blood was drawn from the caudal vessel, with syringes containing Na_2EDTA as anticoagulant, and transferred to ice-cold Eppendorfs containing 1 TIU of aprotinin, a serine-protease inhibitor. The blood was spun at 4 °C for 10 minutes at 13,500 rpm, after which the supernatant plasma was stored in Eppendorf vials and quickly frozen. Fish were

subsequently placed on ice and a piece of head skin and several scales from the left-hand side of the fish were transferred to sterilised Eppendorfs and immediately frozen in dry ice. Samples were stored at -80 °C until RNA isolation.

αMSH determination

The αMSH concentration in the plasma and in the pituitary gland was determined as described by Arends et al. (1999). The antiserum used for the αMSH radio immunoassay cross-reacts for 100% with des-, mono- and di-acetyl αMSH, and was used in a final dilution of 1:60,000. Immunocomplexes were precipitated by 7.5 % (w/v) polyethylene glycol and 2.5 % (w/v) bovine serum albumin. The detection limit was 25.2 pg/ml of sample. Pituitary glands were homogenized mechanically on ice in 100 µl of 0.1M HCl. After centrifugation (13,600 rpm, 4° C) for removing membranes and cellular debris, the supernatant was used to assess the total amount of αMSH in the pituitary glands. To separate the different isoforms of αMSH, reversed-phase HPLC was used as described by Arends et al. (2000). First, a mixture of synthetic des-, mono- and diacetyl αMSH (Sigma) was separated on a Pharmacia µRPC C2/C18 sc 2.1/10 column with ddH_2O/0.1% trifluoracetic acid (TFA) as equilibration eluent and a gradient of acetronitril/0.1% TFA from 0-100% as secondary eluent to determine the elution time of each isoform, and subsequently, the superfusates were fractionated following this protocol. One-minute fractions were collected and the αMSH concentration in these fractions was determined by RIA. To calculate the amount of des-, mono- and diacetyl αMSH, the area under the curve was measured for each form. The sum of the amounts of des-, mono- and diacetyl αMSH was set at 100%. The amount of each individual form was expressed as a part of this 100%.

Melanophore responses

Micrographs were taken from scales of fish from the three different backgrounds prior to the start of the experiments and the degree to which the skin was covered by dark melanophores was assessed (see Chapter 5). These values indicate the initial scale coverage values for each background.

Fifteen minutes prior to the experiment, scales were immersed in physiological saline containing 60 mM KCl. The high concentration of K^+ depolarized the melanophores causing aggregation of the melanosomes. Scales were photographed immediately prior to the start of the experiment, designated the "time 0 melanophore status". Subsequently, scales were immersed in a solution containing αMSH for 30 min after which another micrograph was taken. Des, mono and di-acetylated αMSH (Sigma) were tested at concentrations ranging from 10^{-11} to 10^{-7} M. The melanophore responsiveness was determined by the changes in dispersion state after 30 min of immersion in test solution. We used Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose, USA) to measure the area (pixels) of the scales covered by epidermis and then measured the area (pixels) covered by melanin using the "magic wand" selection tool. By dividing

the two values, a melanophore coverage value expressed as a percentage of the scale surface (epidermis) could be obtained. Changes were initially quantified by assessing the percentage of scale covered by melanophores at time 0 and subtracting these values from the percentage of scale melanophore coverage after 30 min of immersion.

Cloning and sequencing

To obtain the nucleotide sequence of the melanocortin-1-receptor in tilapia, two oligonucleotide primers were designed based on the Fugu MC1R sequence: F1fw, 5'- GGT GGA GAA CAT CCT GGT GAT TCT GG-3'; F3rv, 3'- CGC AGC TCC TGG CTC CGG TAC GCG-3'. Head skin tissue was homogenized in TRIzol reagent (Gibco BRL, Gaithersburg, USA) and RNA was isolated according to the manufacturer's instructions. 1µg of RNA was reverse-transcribed with 300 ng of random primers, 10nmol dNTPs, 200 nmol, 10 U RNase inhibitor and 200 units of RT Superscript II (Gibco BRL) for 50 min at 37 °C. PCR of the above mentioned primers on the obtained cDNA yielded a partial MC1R sequence. The remainder of the sequence was obtained by RACE (rapid amplification of cDNA ends)-PCR (GeneRacer, Invitrogen) according to the manufacturer's protocol, including the use of nested PCR. Gene specific primers used for the RACE-PCR were: MC1RC3fw, 5'- AAC AGG CGC CAG TCC ACA AGT ATG A-3'; MC1RC3nestedfw, 5' - CTG TAA CTC CCT CAT CGA CCC GCT TA-3'; RACE5'fw, 5'- GAT GCT GTG ATA CCT CAG CGC GT-3'; RACE5'nestedfw, 5'- TAG TAC ATG GGC GAG TGG AGG TT-3'.

PCR products were ligated into pCR4-TOPO plasmid vector and transformed into chemically competent TOP 10 *Escherichia coli* cells (TOPO TA cloning kit, Invitrogen, Carlsbad, USA). After selection on LB-kanamycin agar, transformed cells were screened for appropriate size inserts with T3 and T7 primers. Sequence determination was carried out with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, USA).

Phylogenetic analysis

Sequences for other fish melanocortin-1 receptors were retrieved from the NCBI site (www.ncbi.nih.gov), using the Genbank database. Multiple-sequence alignment was carried out with ClustalW at the BioAsp website (www.bioasp.nl) from the Centre of Molecular and Biomolecular Informatics (Nijmegen, The Netherlands). A phylogenetic tree was constructed on the basis of amino acid difference (*p*-distance) with a neighbour-joining method with 1000 bootstrap replications, with MEGA version 2.1 (Kumar et al., 2001; Saitou and Nei, 1987).

MC1R expression

Relative expression of the melanocortin-1 receptor was assessed by quantitative RT-PCR. Head skin or scale tissue was homogenized in TRIzol reagent (Gibco BRL). Total RNA was extracted according to the manufacturer's

instructions and reverse transcribed. On the basis of the tilapia MC1R sequence, the following quantitative PCR primers were designed: MC1fw, 5'- GGA GAC CAT ATT CAT GCT TCT CAA-3'; MC1rv, 5'- ATC ATC ACG TCG ATG ACG TTG T-3'. Reference housekeeping genes used were β-actin and 40S, of which the following primer sets were constructed: BACTfw, 5'- GCC CCA CCT GAG CGT AAA TA-3'; BACTrv, 5'- CCT GCT TGC TGA TCC ACA TCT-3'; 40Sfw, 5'- GAG ATG CTT ACA GGC GAT CTG-3'; 40Srv, 5'- GCC ACC TCT GAA CTG GAA CT-3'.

Five µl of 50 times diluted RT-mix was used as a template in an amplification mixture containing 12.5 µl SYBR Green Master Mix (Applied Biosystems) and 3.75 µl of each primer (final concentration 300 ng). Real-time quantitative PCR was performed on a GeneAmp 5700 (Applied Biosystems). Ct-values were determined and expression of MC1R was calculated as a percentage of β-actin or 40S. All results presented here are expressed relative to 40S, as expression patterns did not vary between 40S and β-actin.

Statistical analyses

Physiological parameters between fish from different backgrounds and differences between the groups in expression of MC1R were assessed by ANOVA, followed by Dunnett's C post-testing when significance was indicated. The differences in percentage of scale coverage by melanophores was arcsine transformed (Sokal and Rohlf, 1995) and then tested with the method described above. Correlations between background and experimental parameters were assessed using Spearman's Rho correlation testing. All parameters are expressed as means ± SD. Statistical analyses were performed with SPSS 12.0.1 statistical software (SPSS Inc., Chicago, USA).

Results

Background adaptation

After 25 days of adaptation to the different backgrounds, a clear difference in skin darkness was visible (Figure 1). Fish on a black background (BBG) had the highest melanophore coverage level (43%), and white background (WBG) fish had the lowest coverage values (10%; $P<0.01$). WBG scale coverage values were also significantly lower than the corresponding values of grey background (GBG) scales ($P<0.05$). The skin darkness of GBG and BBG fish did not differ significantly. In both groups individual variation was quite high. There was a strong positive correlation between the darkness of the background and the coverage level ($P<0.01$, $r^2 = 0.582$). There was no significant difference in the plasma concentrations of αMSH between the fish from the different backgrounds. The ratio of αMSH isoforms in pituitary gland homogenates had not changed following adaptation to the backgrounds (Figure 2). More than half of the αMSH present was mono-acetylated; des and di-acetylated αMSH were present in similar amounts.

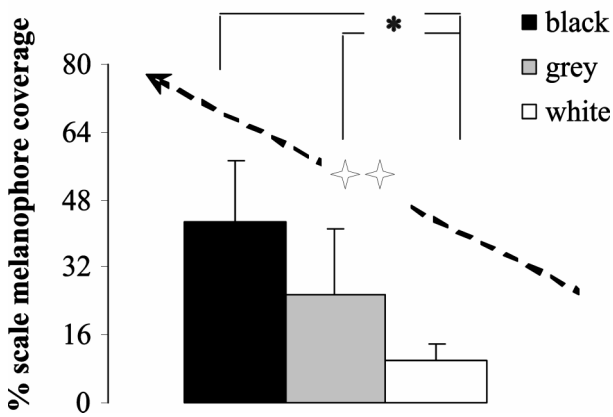

*Figure 1- differences in hue between tilapia from a control grey, black or white background, based on scale coverage by melanophores. Significant differences are indicated by * (P<0.05; n=12 per background). A significant correlation between the darkness of the background and the scale melanophore coverage is indicated by a dotted arrow (✧✧, P<0.01).*

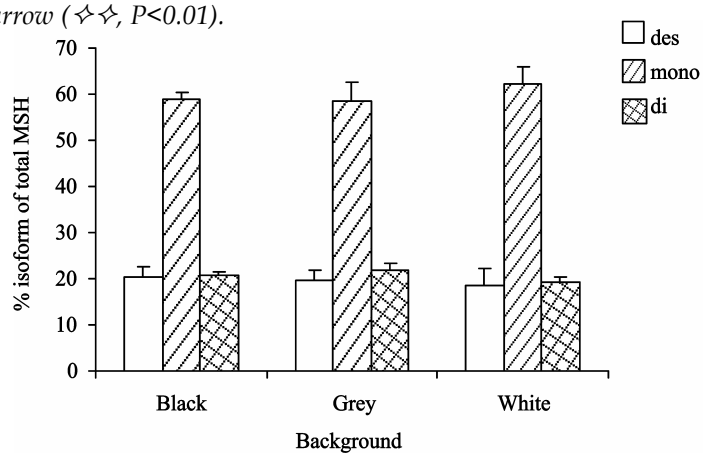

Figure 2- the ratio of αMSH isoforms in pituitary homogenates of background adapted tilapia (n=12 per background).

Response to αMSH isoforms

Overall, scale melanophores responded strongest to mono-acetylated αMSH (P<0.05, Figure 3). Scales from fish from a black background showed the strongest dispersion response, followed by control fish whereas white background fish had the least responsive melanophores. There were no significant differences between the different concentrations of the hormones tested. A significant correlation was found between the intensity of the dispersing response and the darkness of the background (P<0.01, r = 0.159).

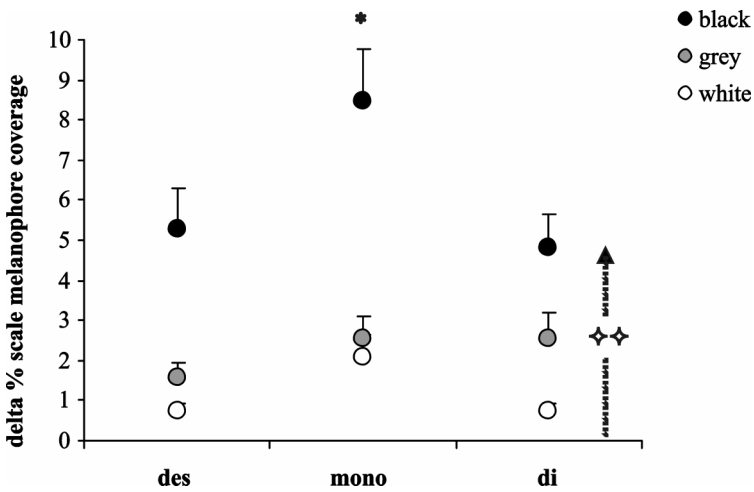

Figure 3- average response after 25 days of adaptation to a grey, black or white background of scale melanophores to different concentrations of des-, mono- and di-acetylated αMSH (concentrations ranging from 10^{-7} to 10^{-11} M; n=8 per background, data ± SEM). The significant correlation between the darkness of the background and the responsiveness is indicated by an arrow (✧✧, P<0.01). The correlation between responsiveness and isoforms was significant for mono-acetylated αMSH (✱, P<0.05).

Melanocortin-1Receptor

Cloning and sequencing of the tilapia MC1R revealed a 1959 bp cDNA sequence with an open reading frame of 325 amino acids (Figure 4, EMBL accession number AJ871147). Amino acid identity is highest to various puffer fish species (74-78%) and zebrafish (68%) and around 50% to various mammalian and avian species (human, 47%; mouse, 50%; dog, 50%; chicken, 55%).

Two potential sites for N-linked glycosylation are present in the N-terminal extracellular domain (Asn^{3} and Asn^{26}) and there are two potential sites for protein kinase C phosphorylation in the intracellular domain between transmembrane regions 5 and 6 (Ser^{223} and Ser^{238}). In this domain a potential cAMP phosphorylation site is also present (Arg^{233}). Figure 5 shows a multiple alignment of the tilapia MC1R with MC1R amino acid sequences of various other species. The seven predicted transmembrane regions are indicated and these stretches show a high degree of conservation between piscine, avian and mammalian sequences.

In Figure 6, a phylogenetic tree is shown for the five different melanocortin receptor genes in various species. This tree was constructed with the neighbour-joining method with 1000 bootstrap replications. The tilapia MC1R clusters together with the fugu MC1R with a reliability of 100%, and together with the zebrafish MC1R these form a distinct cluster within the group of MC1R. This tree also confirms that the obtained sequence is indeed an MC1R and not one of the other four melanocortin receptors.

Figure 4- full-length nucleotide and deduced amino acid sequence of tilapia MC1R cDNA. The deduced amino acid sequence is displayed above the nucleotide sequence. The start codon is boxed in black, the stop codon is indicated by an asterisk (*). Two potential glycosylation sites are boxed in white and two potential PKC phosphorylation sites are boxed in grey. A potential cAMP phosphorylation site is indicated in bold. Two potential adenylation sites are underlined in the 3'-UTR. The EMBL accession number is AJ871147.

```
gaaaagaagcaagctgtaactgcgacagctgatgtagaggcatcctctaagctccagaga          60
cagctgatgaaggttccttactcagtttaaatctgtgctgcagcagcctaaaacccacac         120
                                                    M  E  M  T           4
agagcacctgaaaagctaaaagcttgcagttacctaatagaaggcagacatggaaatgac         180
  N  G  S  L  Q  Y  P  S  I  L  H  A  D  F  G  P  L  N  D  L          24
caacgggtccctgcagtatccctccatacttcacgcggacttcggaccgctaaatgacct         240
  L  E  E  N  E  T  N  S  T  A  G  E  R  N  W  L  N  C  V  Q          44
tctggaggagaacgaaacgaactccaccgcaggagagcgaaactggctgaactgcgttca         300
  I  R  I  P  Q  E  L  F  L  A  L  G  L  I  S  L  V  E  N  I          64
Gatccggatccctcaggagctcttcttggcactgggactcatcagtctggtggaaaacat         360
  L  V  I  M  A  I  I  K  N  R  N  L  H  S  P  M  Y  Y  F  I          84
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ccttatcctcatcctcgcctgccccaccagccccttctgcaactgtttctttcgaaactt        1020
  N  L  F  L  I  L  I  I  C  N  S  L  I  D  P  L  I  Y  A  Y         304
taacctttcctcatcctcattatctgtaactccctcatcgacccgcttatatacgcgta        1080
  R  S  Q  E  L  R  K  T  L  Q  E  M  V  L  C  S  F  C  F  G         324
tcggagccaggagctgcgtaaaaccttgcaggagatggtcctgtgttcgttttgctttgg        1140
  V  *                                                               325
cgtgtgacatcgtggacattcatgaacaaaaccgagcgcaacacaacattcttcctctgt        1200
cttcaacatgtccgaagatagaataagacacaactgatatatgtttaaacatgcagatgt        1260
tgcccgtgcgcgagtgcacatgacagctgaagactctgtcaaactgcaaacatttgatta        1320
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agtatcttaatgacatttttctttaaagaatgatgtgcattgcaagaactcaaaaaata        1740
gaagcagcgaataatctcttagttactgcggctcaaatgcagattattttatatatagac        1800
tgtctttgggaaaatgttcaagcatggtgcaaacggagcagggaacagatgaaacacagc        1860
ttgattggagagggcatcacactaaaccttaaaatgacactgtgtggcaaactcaaaaat        1920
tactgttacaaaaataaatgtcaaatatcccagtgattt                             1959
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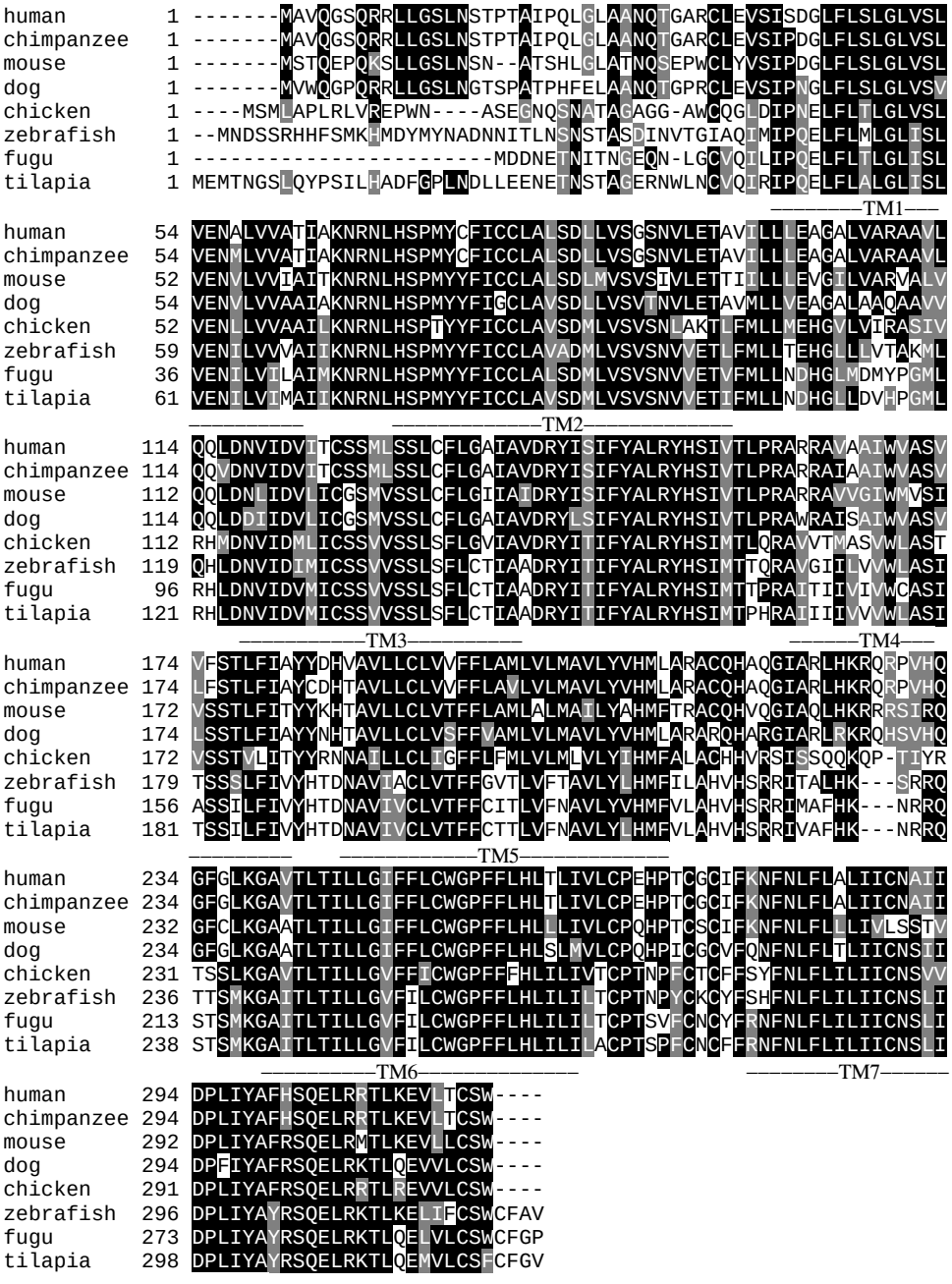
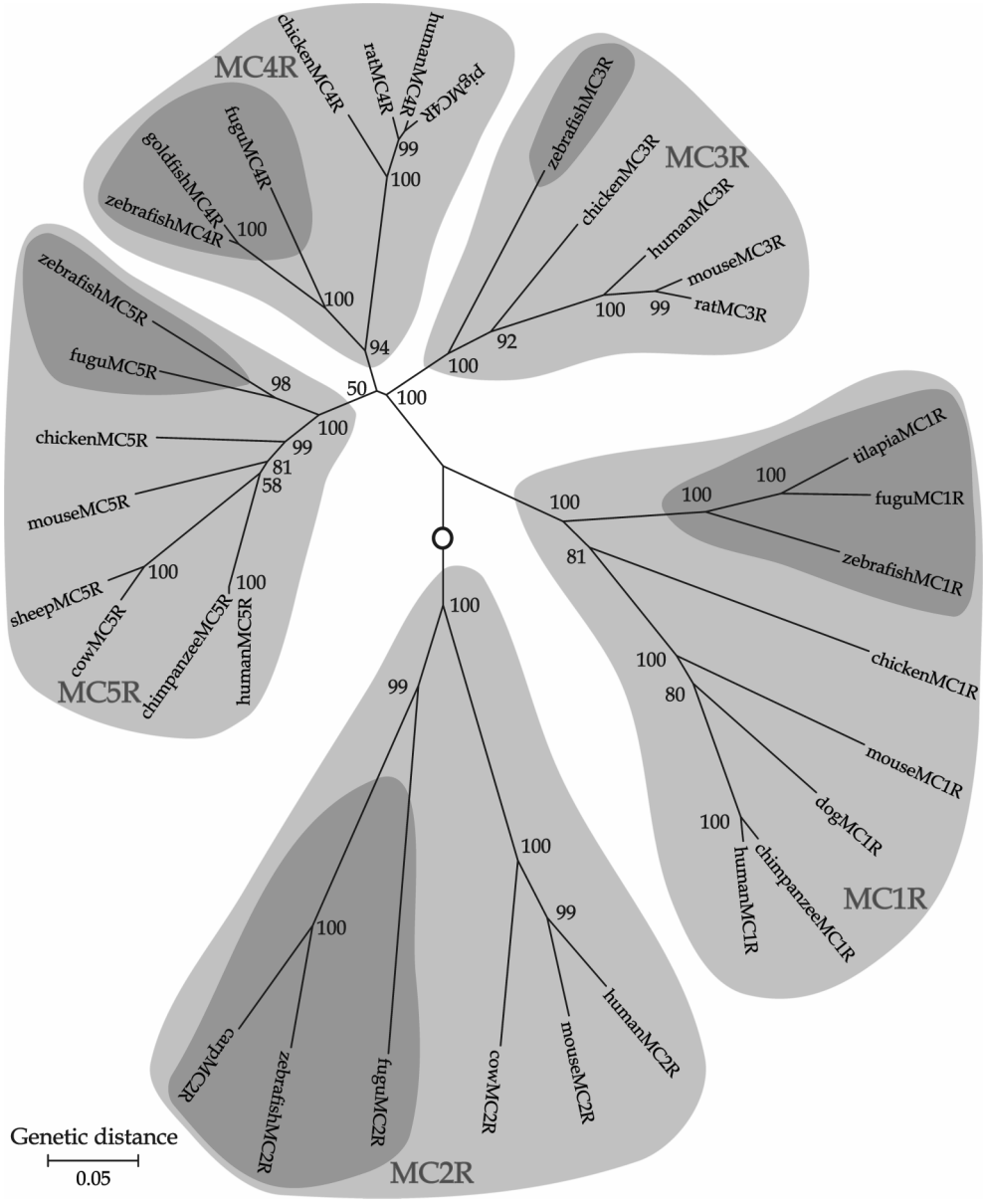
Figure 5- multiple alignment of MC1Rs of eleven different vertebrate species. Hyphens indicate gaps introduced for maximum identity. Identical amino acids are indicated in black boxes, conservative constitutions in grey boxes. The seven predicted transmembrane regions (TM 1 to 7) are indicated. Accession numbers: human (Homo sapiens), Q01726; chimpanzee (Pan troglodytes), Q9TUK4; mouse (Mus musculus), Q01727; dog (Canis familiaris), O77616; chicken (Gallus gallus), P55167; zebrafish (Danio rerio), Q7ZTA3; fugu (Takifugu rubripes), Q7ZSY9 and tilapia (Oreochromis mossambicus), AJ871147.




MC4R
chickenMC4R
ratMC4R
humanMC4R
pigMC4R
fuguMC4R
goldfishMC4R
zebrafishMC4R
MC3R
zebrafishMC3R
chickenMC3R
humanMC3R
mouseMC3R
ratMC3R
MC5R
zebrafishMC5R
fuguMC5R
chickenMC5R
mouseMC5R
sheepMC5R
cowMC5R
chimpanzeeMC5R
humanMC5R
MC1R
tilapiaMC1R
fuguMC1R
zebrafishMC1R
chickenMC1R
mouseMC1R
dogMC1R
humanMC1R
chimpanzeeMC1R
MC2R
carpMC2R
zebrafishMC2R
fuguMC2R
cowMC2R
mouseMC2R
humanMC2R
Genetic distance
0.05

Previous page:

Figure 6- *neighbour joining tree of melanocortin-receptor amino acid sequences. Numbers at branch nodes represent the confidence level of 1000 bootstrap replications. The starting point for cluster formation is indicated by an open circle. Light grey indicates the different MC-receptor clusters and fish species are indicated in dark grey within these clusters. Accession numbers: tilapiaMC1R (Oreochromis mossambicus), AJ871147; fuguMC1R (Takifugu rubripes), Q7ZSY9; zebrafishMC1R (Danio rerio), Q7ZTA3; chickenMC1R (Gallus gallus), P55167; mouseMC1R (Mus musculus), Q01727; dogMC1R (Canis familiaris), O77616; chimpanzeeMC1R (Pan troglodytes), Q9TUK4; humanMC1R (Homo sapiens), Q01726; humanMC2R (Homo sapiens), Q01718; mouseMC2R (Mus musculus), Q64326; cowMC2R (Bos taurus), P34974; fuguMC2R (Takifugu rubripes), Q7ZSX7; zebrafishMC2R (Danio rerio), Q7ZTA2; carpMC2R (Cyprinus carpio), Q6EWJ2; humanMC5R (Homo sapiens), P33032; chimpanzeeMC5R (Pan troglodytes), Q9TT23; cowMC5R (Bos taurus), P56451; sheepMC5R (Ovis aries), P41983; mouseMC5R (Mus musculus), P41149; chickenMC5R (Gallus gallus), O73671; fuguMC5R (Takifugu rubripes), Q7ZT40; zebrafishMC5R (Danio rerio), Q8JGW1; zebrafishMC4R (Danio rerio), Q8JGW3; goldfishMC4R (Carassius auratus), CAD58853; fuguMC4R (Takifugu rubripes), Q90VY0; chickenMC4R (Gallus gallus), Q6E6M6; ratMC4R (Rattus norvegicus), P70596; humanMC4R (Homo sapiens), P32245; pigMC4R (Sus scrofa), O97504; zebrafishMC3R (Danio rerio), Q7ZTA1; chickenMC3R (Gallus gallus), O93259; humanMC3R (Homo sapiens), P41968; mouseMC3R (Mus musculus), P33033; ratMC3R (Rattus norvegicus), P32244.*

Based on the nucleotide sequence as shown in Figure 4, we constructed primers for determination of the expression of the MC1R in skin relative to the expression of the 40S housekeeping gene. There were no significant differences in MC1R expression in the skin between fish adapted to a black or white background compared to control glass background (Figure 7).

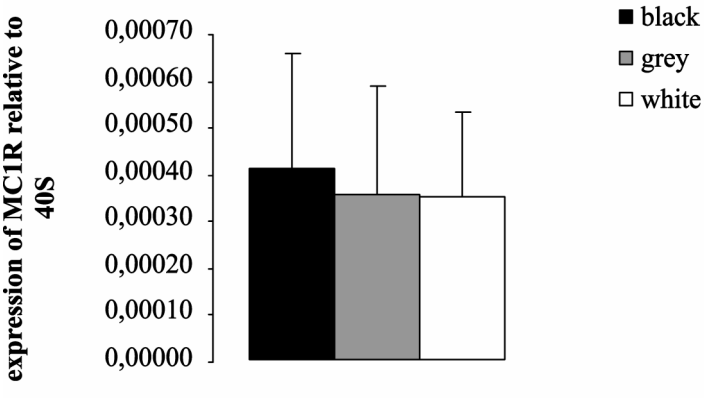


Figure 7- expression of MC1R in skin tissue of tilapia from a control glass, black or white background relative to 40S expression.

Discussion

In this study we present a complete deduced amino acid sequence and partial mRNA sequence for the tilapia melanocortin 1 receptor gene. The expression of this receptor in skin is not influenced by background adaptation to a black or white background and the resulting significant changes in hue. The dispersion of melanosomes within melanophores upon stimulation with αMSH isoforms is significantly higher in black background acclimated fish than in fish from white or grey backgrounds, indicating that the melanophores have become sensitised to αMSH. Mono-acetylated αMSH is the dominant isoform present, and also the most bioactive isoform to stimulate melanosome dispersion, independent of the background.

Background adaptation

Twenty-five days of adaptation to a black or white background resulted in marked and significant changes in the hue of tilapia. In white background tilapia, the lightness of the skin, as seen from the melanophore coverage of scales, was significantly lower than for black and grey background fish. It is generally believed that the regulatory factors involved in initial rapid pigmentation pattern changes (physiological colour change) are also of importance for the longer term (morphological) changes in pigmentation (Fujii, 2000; Sugimoto, 2002). The significant decrease in scale coverage by melanophores is likely to be the result of degeneration by apoptosis of melanophores. Norepinephrin is a likely candidate

to induce this apoptosis; in this context melanin concentrating hormone (MCH) is also a potential apoptosis inducing factor (Sugimoto et al., 2000, 2002). The increase of melanophore cell numbers observed on a black background in fish and amphibians is classically linked to αMSH (Bagnara and Hadley, 1973; Fujii, 2000). However, in the present study no differences were found in plasma αMSH levels between fish from the three different backgrounds. This suggests that at this stage of the background adaptation process, the *in vivo* regulation of skin pigmentation in tilapia is under the control of physiological factors other than αMSH.

Bioactivity of αMSH isoforms

While there is apparently no *in vivo* relation between plasma αMSH levels and the darkness of the skin of tilapia, *in vitro* application of αMSH to scales has a clear dispersing effect on the melanin granules within the melanophores. This suggests once more that in tilapia the endocrine regulation of skin pigmentation includes αMSH, but only as a minor factor. The post-translational acetylation of αMSH indeed seems to affect bioactivity as mono-acetylated αMSH had a stronger dispersing effect on the melanosomes of scale melanophores than desacetyl αMSH. In mammals, des-acetylated αMSH blocks opiate receptor binding stronger than monoacetyl αMSH, whereas monoacetyl αMSH was 10-100 fold more effective at increasing pigmentation, arousal, memory, attention and excessive grooming (see Mountjoy et al., 2003). Rudman et al. (1983) have shown that di- and monoacetyl αMSH have similar melanotropic potency on frog melanophores, whereas desacetyl αMSH had the lowest melanotropic potency. We now find the same for tilapia melanophores.

The bioactivity of αMSH isoforms is determined by the receptor profile of the cell and the combined second messenger pathways. The response to each individual isoform was highest in scales of black background fish. In medaka, *Oryzias latipes*, enzymes that are part of the intracellular signalling pathway are more active in black background melanophores than in melanophores of white background fish. These protein phosphatases decrease the sensitivity of the melanophores to externally administered cAMP and black background adapted medaka required more cAMP than white background fish (Sugimoto, 1993; Sugimoto et al., 1997). The higher sensitivity to cAMP observed in melanophores of white background adapted medaka facilitates a quick adaptive response should the background darken (Sugimoto et al., 1997). A similar situation may be found in background acclimatised tilapia as well. Circulating levels of αMSH show no differences between black, grey and white background adapted fish, while the black background fish show a higher responsiveness to αMSH than grey and white background fish. The possibility exists that αMSH turnover could be higher at constant plasma levels, but this was not further analysed. Rather than changes in plasma αMSH levels upon background adaptation, the sensitivity of the αMSH receptor in melanophores may be increased on dark

backgrounds and reduced on light backgrounds. Upon binding of αMSH to its receptors, intracellular cAMP levels rise. Enhanced αMSH binding may therefore stimulate higher intracellular levels of cAMP and in this way compensate for the reduced sensitivity to cAMP as described by Sugimoto and co-workers (1997).

Mono-acetylation apparently stimulates the potency of αMSH whereas di-acetylation may reduce the activity. According to Mountjoy and co-workers (1999, 2003) the acetylation state of αMSH is the key factor for its bioactivity: for some functions acetylation of αMSH inhibits its bioactivity, and for other functions monoacetyl αMSH is more potent than the des-acetylated form. Di-acetylated αMSH has been indicated to be the isoform with corticotropic potency (Balm et al., 1995; Lamers et al., 1992), and Rudman and colleagues (1983) have shown that the higher the acetylation-state, the longer it takes for the peptide to be degraded. The prolonged presence of the di-acetylated αMSH isoform may compensate for its reduced melanotropic bioactivity.

Melanocortin-1 receptor

The MC1R is known from mammalian literature to be involved in the regulation of skin pigmentation (Ha et al., 2003; Kijas et al., 1998) and is considered the main receptor for melanotropic actions of αMSH. The high degree of homology between the piscine MC1R sequences and those in amphibians, birds and mammals indicates that this function has been conserved throughout the evolution of the vertebrates. We therefore assume that the MC1R is also the main receptor for αMSH binding in tilapia to serve melanotropic actions. The expression of the tilapia MC1R does not differ between tilapia adapted to different backgrounds. We have postulated earlier that a change occurs in the intracellular signalling system following adaptation to a black background, rather than an increase in receptor expression. An affinity change in the receptor seems unlikely as our nucleotide sequence was derived from mRNA originally obtained from head skin of black and white as well as glass background adapted fish. The cDNA was identical for the clones we obtained from each background.

Interestingly, affinity studies on the MC1R in fugu show that, unlike that of mammals, the fugu MC1 receptor does not have the highest affinity for αMSH. The affinity of the MC1R for ACTH appeared to be 10 times higher than for αMSH (Klovins et al., 2004). These authors postulate the hypothesis that the MC1R has evolved from a receptor with a preference for ACTH in early vertebrates to one that has specific affinity for αMSH in mammals. These findings also point to a possible role for ACTH in the regulation of pigment dispersion in fish as has been demonstrated by Fujii (1993, 2000). Indeed, initial tests in our laboratory with ACTH have shown that this peptide also has melanotropic bioactivity on scale melanophores of tilapia (unpublished results).

The main findings of our study are that the MC1R receptor is present in tilapia skin and shows high homology with various other vertebrate species and that this receptor is not differentially up-regulated in fish adapted to different

backgrounds. We hypothesize that αMSH binds to this receptor to invoke melanosome dispersion. Acetylation of des-acetylated αMSH stimulates the melanotropic potency of the peptide and fish on a black background show the highest response to all αMSH isoforms. The increased potency of αMSH either by acetylation or by previous background adaptation is not mediated by increased receptor expression, but may result from receptor affinity changes or from adaptive changes in the intracellular signalling system. Indeed, while plasma αMSH levels are unchanged between fish from the three different backgrounds used in this study, the response to αMSH is different. This indicates an increased sensitivity to αMSH in black background acclimated fish.

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Chapter 7

General Discussion

In this thesis, the function of α-melanophore stimulating hormone (αMSH) in the control of skin pigmentation and the stress response was studied. This included *in vivo* and *in vitro* studies on synthesis and bioactivity of the three isoforms of αMSH (des, mono and di-acetylated), correlation studies with other hormones and peptides during stress responses and skin colour change, and receptor studies to address the question of the regulation of skin melanophores by αMSH. The main findings presented are:

- ✧ The regulation of αMSH release from the pituitary gland is similar in tilapia (*Oreochromis mossambicus*) and in red porgy (*Pagrus pagrus*): hypothalamic TRH and CRH can stimulate the release of αMSH, and dopamine (DA) and MCH can inhibit this release. Moreover, in cultured (malpigmented) red porgy this regulation does not differ from the regulation in wild red porgy.
- ✧ In both red porgy and in tilapia, no correlation between plasma αMSH levels and the darkness of the body was found.
- ✧ During exposure to an acute stressor, αMSH plasma levels rise. In red porgy, chronic crowding stress did not result in increased plasma levels. In tilapia, chronic exposure to water with low pH caused elevated plasma αMSH levels. A rise in plasma αMSH levels seems to depend on the type of stressor used.
- ✧ *In vitro* studies on scale melanophores show that αMSH does induce melanin dispersion in tilapia melanophores. Mono-acetylated αMSH is more potent than the des and di-acetylated isoforms.
- ✧ Expression of the tilapia melanocortin-1-receptor is not influenced by adaptation to a black or white background.

These findings will be discussed below and red porgy and tilapia responses to different backgrounds and different stressors will be compared, with particular focus on the function of αMSH.

Pigmentation

Melanophore-stimulating hormone, as the name implies, is classically known for its ability to stimulate dispersion of melanin granules within the melanophores of fish, amphibians and reptiles (Bagnara and Hadley, 1973). αMSH is also involved in the proliferation of new melanophores and in melanin synthesis in all vertebrate classes (Fujii, 2000), and these are the main functions of the hormone in birds and mammals. Also, the structure of αMSH (a tridecapeptide) is highly conserved throughout the vertebrate kingdom (Arends et al., 2000).

In a number of fish species, circulating αMSH levels are elevated in dark pigmented animals compared to conspecifics with a light skin. These differences in pigmentation can be achieved by supplying the fish with a dark (black) or light (grey or white) background, upon which sharks and fish will launch a

background adaptation response. These species include dogfish, eel, trout and red drum (Baker et al., 1984; Rodrigues and Sumpter, 1984; Zhu and Thomas, 1996).

In both species of fish studied in this thesis (red porgy and tilapia) a correlation between plasma αMSH levels and the darkness of the skin was absent. Background adaptation induced a clear and significant difference in the lightness of the skin of fish adapted to dark (black or dark-red) or light (grey or white) backgrounds but, in all these studies (described in Chapters 3 to 6) plasma αMSH levels were never significantly correlated with the darkness of the skin. This indicates that the malpigmentation observed in cultured red porgy may not be related to αMSH. Indeed, studies on the regulation of αMSH release in this species indicated that while the data-set for wild red porgy (unpublished results) was far less extensive than that for the cultured fish (Chapter 2), for each of the hormones tested (TRH, CRH, DA, MCH) the effects did not differ between wild and cultured red porgy. In a parallel study within the EU-consortium, light microscopic analysis of the skin of red porgy indicated that the erythrophores or red pigment cells were almost absent in cultured fish, whereas they were abundant in wild fish (A. Sterioti, personal communication). Recent research on fish feed composition has indicated that the reddish colour of the red porgy can be restored by adding more sources of carotenoids to the diet (Cejas et al., 2003). Shrimps and mussels are the natural food source for red porgy and apparently the red colour of the skin is obtained from these types of food.

A number of other fish species besides red porgy and tilapia also show a lack of correlation between the darkness of the skin and the plasma levels of αMSH. Flounder, arctic charr and the gilthead sea bream are among these species of teleost fish (Arends et al., 2000; Baker et al., 1984; Höglund et al., 2002). From a phylogenetic point of view, the species mentioned earlier (e.g. dogfish, eel and trout) that do show a correlation between body darkness and circulating αMSH levels are older fish species than flatfish, sea bream and cichlid fish (Amores et al., 2004). We speculate that during the course of evolution in fish, the involvement of αMSH in the *in vivo* regulation of skin pigmentation is decreasing in favour of other melanogenic hormones or neurotransmitters. Alternatively, αMSH is known to be involved in control of cortisol production and centrally in satiation signalling, in line with its general pleiotropic nature.

Melanophores

Interestingly, while an *in vivo* correlation between plasma αMSH levels and the darkness of the skin is absent, *in vitro* application of αMSH did induce melanosome dispersion in tilapia melanophores (Chapter 6). A similar finding was reported for isolated melanophores of skin sections in flounder (Burton and Vokey, 2000). These findings suggest that while αMSH has the ability to cause pigment granule dispersion *in vitro*, this action does not seem to prevail *in vivo*.

Apparently, pigmentation changes in species such as flounder, red porgy and tilapia are under the control of factors other than αMSH. The regulation of melanin granule (or melanosome) motility by various substances has been reviewed by Fujii (2000). Among potential candidates are MCH, melatonin, somatolactin (SL) and catecholamines, especially norepinephrine. MCH was shown to be more potent than αMSH in an *in vitro* competitive assay on flounder melanophores (Burton and Vokey, 2000). In tilapia, fish that were kept on a white background showed elevated MCH concentrations in the hypothalamus (Gröneveld, 1995). Interestingly, the agouti-protein, an endogenous αMSH-antagonist in mammals (Mountjoy et al., 1999), has recently also been discovered in goldfish, *Carrassius auratus*, and in zebrafish, *Danio rerio* (Cerda-Reverter et al., 2003; Song et al., 2003). This could be another candidate for the control of skin pigmentation in fish.

Table 1- the response of tilapia scale melanophores (from a control glass stock tank) to various hormonal and neuronal substances after 30 min of incubation. Alpha-adrenoceptor agonists induce aggregation while a β-adrenoceptor agonist induces dispersion. From the various hormonal substances, only MCH evokes aggregation. The change in melanophore scale coverage is expressed as fold-change compared to the status at time 0 (van der Salm, unpublished).

Substances tested	Concentration (log M)	Fold change	Direction of change
Desacetyl αMSH	-6	1,27	Dispersion
Monoacetyl αMSH	-6	1,5	Dispersion
Diacetyl αMSH	-6	1,4	Dispersion
βMSH	-6	1,2	Dispersion
ACTH	-6	1,25	Dispersion
MCH	-6	-1,93	Aggregation
β Endorphin	-6	1,25	Dispersion
N-acetylated β End.	-6	1,78	Dispersion
Phenylepinephrine (α1-selective agonist)	-7	-1,5	Aggregation
Clonidine (α2-selective agonist)	-7	-1,8	Aggregation
Metaproterenol (β2-selective agonist)	-7	1,2	Dispersion

Pilot studies on scale melanophore responsiveness in tilapia show that besides the three isoforms of αMSH tested in Chapter 6, a great number of other substances, hormonal as well as neuronal (targeted against α and β adrenoreceptors), can affect the dispersion state of melanophores (Table 1). These findings particularly point to MCH as a very potent regulator of the melanophore

dispersion state. Also, the responses to nervous substances (catecholamines and related agonists) are generally stronger than the responses to hormonal regulators.

Stress responses

The malpigmentation of cultured red porgy initially raised the question of whether this darkness was the result of stress-associated elevated plasma αMSH levels. As previously described, involvement of αMSH could not be demonstrated. However, during exposure of red porgy to an acute stressor (netting combined with air exposure, Chapter 4) and of tilapia to a single chronic stressor (acidified water, Chapter 5) plasma αMSH levels did rise. These increases were found for fish from all backgrounds used in both experiments, and were therefore not related to the darkness of the body.

The involvement of αMSH in the stress response has been considered before in mammals (*e.g.* Lindley et al., 1990) and in several species of fish. Plasma αMSH levels in fish can rise after handling, confinement, air exposure, thermal shock and during chronic exposure to acidified water (Arends et al., 2000; Lamers et al., 1994; Sumpter et al., 1985). The increase in plasma αMSH levels upon exposure to stressors would indicate a physiological role for αMSH in the response to those stressors. Such a function was proposed by Lamers et al. (1992), who suggested αMSH to have corticotropic potency. Particularly di-acetylated αMSH could stimulate the release of cortisol from the interrenal tissue in tilapia, a function that may be potentiated by simultaneous stimulation with β-endorphin (Balm et al., 1995). An increase of plasma cortisol levels is generally accepted to indicate a stress response. During acute stress, plasma levels can increase 10-fold or more, while during chronic stress cortisol levels remain elevated above basal levels (up to 20 ng/ml; Wendelaar Bonga, 1997). In vertebrates, the regulation of cortisol release is generally accepted to be under control of adrenocorticotropic hormone (ACTH), as described in Chapter 1. POMC derived peptides such as αMSH and ACTH can bind to melanocortin receptors. For αMSH to have corticotropic potency next to ACTH, this would imply the presence of a melanocortin receptor in the head kidney of fish that, during low pH stress, or perhaps also under the influence of other stressors, would increase the binding preference for αMSH relative to ACTH. As yet, there is no evidence that any melanocortin receptor present in the head kidney is up-regulated in this way during stress. Recent studies in our lab (J.R. Metz, personal communication) show that carp express specifically MC2R in cortisol-producing cells in the head kidney. Maybe this receptor is less specific for ACTH than its mammalian counterpart. Alternatively, other MC receptors could be expressed in these interrenal cells. However, the likely candidate MC5R was not found in carp head kidney.

Recent research is now also pointing to a role for αMSH in food intake and in immune responses (Cerda-Reverter et al., 2003; Harris and Bird, 2000). This implies that αMSH affects secondary stress responses (energy mobilization, immune functions) rather than the primary stress response that results in cortisol release (Wendelaar Bonga, 1997). To address the question of what exactly is the function of αMSH in fish during stress, the use of molecular tools to study receptor localization, expression and affinity may yield some answers.

Melanocortin receptors

Melanocortin (MC) receptors were first identified in humans (Cone et al., 1996), and were later also found in other mammals and in birds, with remarkable cDNA synteny (Schiöth et al., 2003). MC receptors are a family of G-protein-coupled receptors that can bind proopiomelanocortin (POMC)-derived peptides (*e.g.* ACTH, α, β, γ and δ-MSH). Recent research has shown that all five tetrapod receptor subtypes (MC1R to MC5R) are present in fish as well (Haitina et al., 2004; Klovins et al., 2004). In mammals, αMSH binds with the highest affinity to the MC1R. In fish however, these binding affinities may be different (Klovins et al., 2004). Research on Japanese pufferfish, *Takifugu rubripes*, and rainbow trout, *Oncorhynchus mykiss*, has shown that in both species most of the MC-receptors have a higher affinity for ACTH than for αMSH (Haitina et al., 2004; Klovins et al., 2004). The cDNA sequence of the tilapia MC1R that we presented in Chapter 6 was obtained with primers designed on the basis of the cDNA sequence of the pufferfish MC1R. The tilapia MC1R has 74-78% homology with pufferfish MC1R. This indicates that the affinity profile of the tilapia MC1R may be similar to that of the pufferfish MC1R. The studies described above (Table 1) indicate that the affinity of this receptor for ACTH is similar to that for des- and diacetyl αMSH, while in pufferfish, ACTH affinity was 10-fold higher than monoacetyl αMSH affinity (Klovins et al., 2004). The bioactivity of peptides is not solely determined by the receptor affinity; in Chapter 6 we proposed that changes in the intracellular signalling pathways were involved. Peptides that exhibit preferable binding are not necessarily the most bioactive peptides.

With these remarks in mind, it would be very interesting to investigate the affinity profiles of the MC receptors expressed in the skin of different species of fish and to use selective agonists to address the resulting melanotrope effect of each receptor. The importance of αMSH as a colour changing agent via the MC1R may be a function that is not as pronounced in fish. Indeed, Klovins and colleagues (2004) suggest that the MC1R may have evolved from an ACTH preferring receptor in fish to a specific αMSH receptor in mammals. An explanation may be found for these different responses by comparing the affinity profiles between fish that do show a correlation between plasma αMSH levels and pigmentation and those that do not.

Conclusions and perspectives

In this thesis, two species of fish have been studied to analyse the role of αMSH in the regulation of skin pigmentation and in the stress response. The lack of correlation between plasma αMSH levels, both in red porgy and tilapia, and the darkness of their skin implicates that *in vivo* it is not αMSH that is the main pigment regulating factor in these fish. While hormones may show clear effects when applied *in vitro*, this is only an indication of a function of these signals as there are many other hormones and neurotransmitters than can also exhibit or affect this function and may be more potent at doing so.

The function of a hormone is effected by a number of factors. For αMSH, the following steps occur, from its synthesis to stimulation of the target. Upon synthesis, acetylation, glycosylation and/or amidation can modify the structure and thereby the bioactivity of the peptide. Release of the hormone can be influenced by multiple messengers (see Chapter 2). During transport via the blood the hormone can be degraded faster or slower depending on the acetylation state (Rudman et al., 1983). When the target is reached, *e.g.* the melanophore, αMSH binds to the receptor with a certain affinity. The expression of the receptor may be altered by stressors, by the background and by numerous other influences. Finally, the intracellular response to binding of the hormone may vary between cells and can be affected by background (Sugimoto et al., 1997).

Therefore, to completely address the main function and bioactivity of αMSH, all these aforementioned steps should be taken into account. By solely focussing on affinity profiles, preferential binding can be indicated, yet changes in intracellular signalling systems can be of importance as well. Increased expression of the receptors can enhance the biological effects of the hormone. Radioactive labelling of αMSH upon synthesis in the pars intermedia of the pituitary gland enables a detailed follow up through the body to localise the targets of the hormone. When these targets have been identified, the relevant melanocortin receptors can be determined and the aforementioned studies on hormone-receptor interaction and the resulting biological effect can be performed (see Figure 1).

So far, there are indications that during stress in fish, αMSH may be involved in the changes in neural circuits that control food intake (and thereby energy metabolism) that occur in order to cope with the stressor, and that the hormone is interacting with the immune system. A direct primary effect within the HPI-axis remains under debate but may be elucidated with the use of the tools mentioned above.

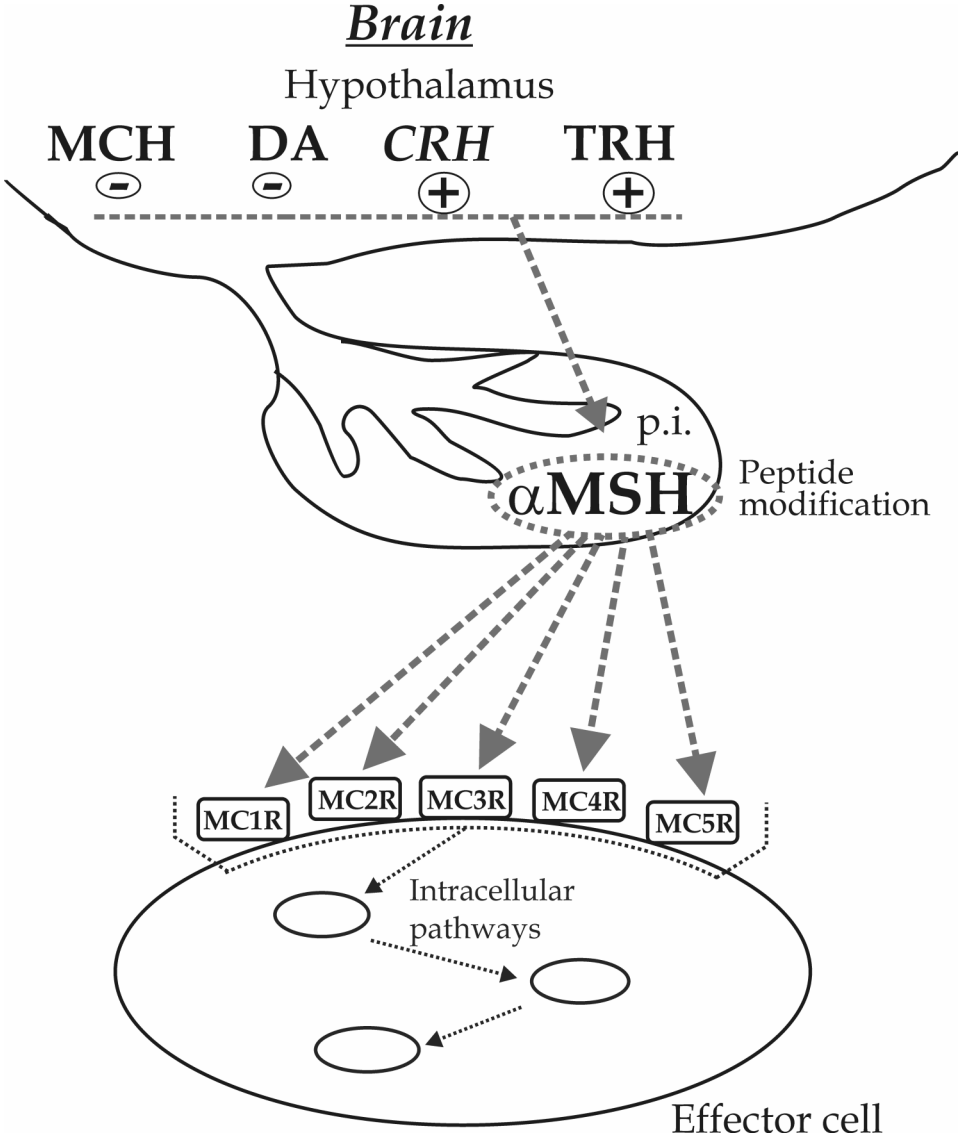

Figure 1- the pathways involved in the functions of the pleiotropic hormone αMSH. Upon synthesis in the pars intermedia (p.i.), desacetyl αMSH can be acetylated once (to yield monoacetyl αMSH) or twice (diacetyl αMSH). The release of αMSH can be stimulated by TRH and CRH and inhibited by MCH and DA. αMSH is transported via the blood to reach an effector cell (e.g. the melanophore, interrenal cells). αMSH can bind to any of the five MC receptors (perhaps excluding the MC2R). The affinity and the expression of these receptors may be altered during stress. Finally, within the cell the signalling pathway may become sensitised, for example in melanophores during adaptation to a black background, or desensitised. Effector cells may be peripheral macrophages, chromaffin cells, interrenal cells, melanophores and other chromatophores, and neuronal circuits in the central nervous system.

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Samenvatting

Voortdurende veranderingen in de omgeving hebben dieren een groot vermogen gegeven om zich daaraan aan te passen en zodoende te kunnen overleven. Nadat het dier een prikkel heeft gekregen dat er in de omgeving iets veranderd is, brengt het een arsenaal aan fysiologische processen op gang om zichzelf zo goed mogelijk aan die betreffende verandering te adapteren. Welke processen dat precies zijn, hangt in grote mate af van de aard van de verandering. De ontvangen prikkel wordt verwerkt in de hersenen, die dan via het perifere zenuwstelsel een snelle reactie teweeg kunnen brengen. Daarnaast wordt er een wat langzamer proces op gang gebracht waarbij de hersenen via een belangrijke endocriene klier, de hypofyse, hormonen af geven aan het bloed. Deze hormonen kunnen perifeer allerlei effecten uitoefenen, waarvan een heel belangrijk effect is de afgifte van cortisol door de kopnieren (bij vissen) of de bijnieren (bij zoogdieren). Dit hormoon behoort, met adrenaline en noradrenaline uit de kopnieren, tot de belangrijkste hormonen van de stress-respons; de reactie op schadelijke of bedreigende veranderingen in de omgeving. De cortisolconcentratie in het bloed kan hierbij langdurig verhoogd zijn.

In dit proefschrift werd het hormoon α-melanophore-stimulerend-hormoon (αMSH) onderzocht, dat wordt aangemaakt in de pars intermedia van de hypofyse. Dit hormoon is betrokken bij een groot aantal adaptieve processen, maar is vooral bekend door het effect op bepaalde pigmentcellen in de huid, de melanophoren. Dit zijn cellen die melanine bevatten, een pigment wat zwart of bruin-rood kan zijn in warmbloedige dieren maar in koudbloedige dieren zoals vissen alleen maar zwarte pigmenten bevat. αMSH is in staat om zich via een receptor (de melanocortine receptor 1; MC1R) aan melanophoren te binden, waarop de melanine granula (melanosomen) in de cel zich gaan verdelen over de vele uitlopers. Dit geeft de cellen een donker, stervormig uiterlijk en dit proces wordt dispersie genoemd. Het omgekeerde proces kan ook optreden. Hierbij migreren de melanine granula juist naar de kern van de cel en wordt de huid lichter: aggregatie (Figuur 1, blz. 11).

αMSH is ook in staat om processen aan te sturen die een rol spelen bij de reactie op stressoren. Stressor is de term die wordt gebruikt om de factor aan te wijzen die in de omgeving veranderd is en een stress-respons opwekt. In vissen is gebleken dat αMSH het metabolisme kan beïnvloeden, een rol speelt bij de afweerreacties van het immuunsysteem en mogelijk zelfs de afgifte van cortisol kan stimuleren.

In dit proefschrift worden bij twee verschillende soorten vissen de processen van achtergrondadaptatie en van stressadaptatie onderzocht en wordt gekeken naar de rol die αMSH daarbij speelt. Vissen zijn ideale modellen omdat via het water de effecten van verschillende stressoren heel specifiek kunnen worden getest. In dit proefschrift is gewerkt met twee vissoorten die beide van belang zijn voor de visteelt: red porgy, een zeewater soort die ook wel gewone

zeebrasem genoemd wordt, en tilapia, die in zoetwater leeft. Beide soorten zijn in staat om de kleur van hun huid snel aan te passen aan die van de achtergrond. Vooral in tilapia is al veel onderzoek gedaan naar de rol van αMSH bij stress. Bij het grootschalig kweken van red porgy doet zich een verschijnsel voor dat een rol voor αMSH doet vermoeden: gekweekte vissen hebben een donkere huid vergeleken met wilde vissen, die een roze-zilverachtige kleur hebben (zie de foto's op blz. 33). Deze donkere kleur maakt de vissen ongeschikt voor de verkoop. Mogelijk hangt de donkere kleur samen met de stress die vissen vaak ondervinden wanneer ze op grote schaal worden gekweekt.

Na een algemene inleiding (*hoofdstuk 1*) wordt in *hoofdstuk 2* gekeken naar de regulatie van de afgifte van αMSH door de hypofyse in de red porgy. Hiertoe werd de hypofyse geïsoleerd uit de vis en in een superfusie opstelling geplaatst. Hierin kan de hypofyse aan verschillende stoffen worden blootgesteld en kan in de daaruit afgescheiden vloeistof de concentratie afgegeven αMSH worden bepaald. Het blijkt dat in red porgy de afgifte van αMSH vooral wordt gestimuleerd door thyrotropin releasing hormone (TRH) en in hoge concentraties ook door corticotropin releasing hormone (CRH). Dopamine (DA) en melanine concentrerend hormone (MCH) hebben beide een remmend effect op de afgifte van αMSH. Al deze stoffen komen in de hersenen van deze vissen voor en worden waarschijnlijk gebruikt om de afgifte van αMSH te reguleren. Eerder onderzoek aan tilapia heeft uitgewezen dat in die soort dezelfde vorm van regulatie aanwezig is. αMSH kan, voordat het door de melanotrope cellen in de hypofyse wordt afgegeven, geacetyleerd worden. In red porgy en in tilapia is monoacetyl αMSH (één acetyl groep) de vorm die het meest wordt afgegeven. αMSH kan daarnaast ook niet geacetyleerd zijn (desacetyl αMSH) of twee acetyl groepen hebben (diacetyl αMSH). De biologische activiteit van αMSH kan beïnvloed worden door het aantal acetyl-groepen dat aanwezig is.

In *hoofdstuk 3* is voor een groot aantal factoren die betrokken zijn bij het kweken van vissen, gekeken naar het effect op de stress respons in de red porgy. Het blijkt dat verschillende achtergrondkleuren (zwart, rood en wit) geen stress oproepen bij het dier. Wel kan de achtergrondkleur de lichaamskleur van de red porgy beïnvloeden. Om de kleur objectief te kunnen meten hebben we een apparaat gebruikt wat kleur opdeelt in een licht-donker component (de L*-waarde), een helderheidcomponent (chroma) en een waarneembare kleurcomponent (de hue; figuur 2, blz. 41). Het bleek dat op een witte achtergrond de L*-waarde van de red porgy toeneemt tot waarden die in wilde vissen ook te vinden zijn. Blauw licht kon deze kleuraanpassing versterken, in tegenstelling tot volledig spectrum (wit) licht. De lichtintensiteit had geen invloed en een hoge dichtheid van de vissen had een negatief effect op de kleur van de huid. De kleurveranderingen gingen niet gepaard met veranderingen in de bloedspiegel van αMSH. Daarnaast had een hoge visdichtheid, wat vaak als chronische stress wordt ervaren door vissen, geen stress respons als gevolg. Dit was eerder in andere onderzoeken wel gevonden. We postuleren hier de hypothese dat het aantal vissen per eenheid water een belangrijkere factor is in

het veroorzaken van een stress respons dan het gewicht aan vissen per eenheid water.

In *hoofdstuk 4* is gekeken naar de stress respons van red porgy die werd opgewekt door een sterke en plotselinge stressor: hierbij werden de vissen vijf minuten lang in een net boven het water vastgehouden. Dit werd door de vissen inderdaad als zware stressor ervaren; geconcludeerd uit de cortisol niveaus in het plasma die vijf tot tien keer hoger waren ná de stressor dan ervoor. Ook de plasma αMSH niveaus waren sterk gestegen na het toepassen van de stressor. De blootstelling aan lucht resulteert in een zuurstoftekort in het bloed wat hogere melkzuur (lactaat) waarden veroorzaakt en waardoor de pH van het bloed daalt.

Vanaf *hoofdstuk 5* wordt het onderzoek verlegd naar tilapia. In deze soort werd gekeken naar het effect van verzuring van het water wanneer dit gelijktijdig met het proces van achtergrondadaptatie plaats vond. Ook werd gekeken naar het effect van water verzuring wanneer deze plaats had nadat achtergrondadaptatie had plaatsgevonden. Het blijkt dat wanneer verzuring gelijktijdig plaats vindt met achtergrondadaptatie, het laatste proces hierdoor bemoeilijkt wordt. Vissen op een witte of glazen achtergrond blijven iets donkerder in verzuurd water, terwijl vissen op een zwarte achtergrond juist iets lichter blijven dan de corresponderende vissen in neutraal water. Het gelijktijdig toepassen van een nieuwe achtergrondkleur èn verzuring van het water resulteerde in een minder sterke stress respons dan het toepassen van verzuring nadat de vissen 25 dagen ongestoord hadden kunnen adapteren. Dit wijst erop dat bepaalde achtergrondkleuren door tilapia als milde stressor kunnen worden ervaren (waaronder zwart) en dat een combinatie van milde stressoren tot een minder sterke fysiologische respons leiden dan wanneer deze na elkaar worden toegepast. Ook in tilapia bestaat er geen correlatie tussen de kleur van de huid en de bloedspiegel van αMSH. Plasma αMSH niveaus nemen wel toe, op alledrie de achtergronden, nadat het water is verzuurd.

De bioactiviteit van de verschillende isovormen (des, mono en di geacetyleerd) van αMSH is getest in *hoofdstuk 6*. Het blijkt dat melanophoren van vissen geadapteerd aan een zwarte achtergrond de sterkste dispergerende reactie vertonen op αMSH, bij elke geteste isovorm. Daarnaast is monoacetyl αMSH de meest potente isovorm om dispersie van melanosomen te veroorzaken voor vissen die zijn geadapteerd aan een witte, zwarte of neutrale (glas) achtergrond. Om de bioactiviteit van αMSH te kunnen relateren aan de aanwezigheid en expressie van de MC1receptor, moest deze voor tilapia eerst gekarakteriseerd worden. Met behulp van moleculair-biologische technieken werd de sequentie van de receptor gevonden en de expressie ervan bepaald in de huid van tilapia die 25 dagen waren geadapteerd aan een zwarte, witte of neutrale achtergrond. Er was geen verschil in expressie tussen deze dieren.

Bij de twee in dit proefschrift onderzochte vissoorten blijkt er geen correlatie te bestaan tussen de niveaus van αMSH in het bloed en de pigmentatie van de huid. Daarentegen heeft αMSH *in vitro* wel degelijk een dispergerend effect op de melanine granula in de melanophoren op de schubben van tilapia.

Dit wijst erop dat αMSH weliswaar de potentie heeft om de huid van de red porgy en van tilapia donkerder te maken, maar dat *in vivo* dit effect wordt overheerst door dat van andere hormonen of neurotransmitters, die een sterker effect hebben op de huidskleur. In het bijzonder adrenaline en noradrenaline kunnen via het zenuwstelsel zeer snel naar de melanophoren worden geleid en kunnen, afhankelijk van het receptorsubtype waar ze aan binden, leiden tot dispersie of aggregatie. Daarnaast zijn er gegevens die er op wijzen dat MCH een sterker effect heeft op de melanophoren dan αMSH.

Iedere verandering in de omgeving is een potentiële stressor, waarbij de sterkte van de respons afhangt van de mate en duur van de stressor. Bepaalde stressoren leiden wel tot een verhoging van het αMSH in het bloed, terwijl andere geen effect hebben. Mogelijk leiden alleen intense stressoren tot een verhoging van αMSH niveaus. De suggestie dat αMSH de afgifte van cortisol kan stimuleren wordt niet ondersteund met de experimenten in dit proefschrift. Om wat meer inzicht te krijgen in de precieze functie van αMSH in de stress respons kunnen moleculair-biologische technieken van pas komen. De expressie van de MC1receptor werd niet beïnvloed door de achtergrondaanpassing, wat aangeeft dat het verschil in gevoeligheid voor αMSH waarschijnlijk via andere wegen tot stand is gekomen. Het is mogelijk dat het proces wat in de cel plaats vindt als reactie op het binden van αMSH kan worden beïnvloedt door veranderingen in de achtergrond.

Er zijn bij de gewervelde dieren tot nu toe vijf verschillende melanocortine receptoren gevonden. Ook bij verschillende vissoorten komen deze voor. Het lokaliseren van de vijf melanocortine receptoren in beide vissoorten en daarbij ook het vaststellen van een affiniteitsprofiel (bindt αMSH sterker aan de betreffende receptor dan bijvoorbeeld adrenocorticotroop hormoon; ACTH), kan antwoord geven op de vraag waar αMSH precies een rol bij speelt. Het hormoon kan tijdens de synthese in de hypofyse (radioactief) gelabeld worden, waardoor gevolgd kan worden waar het hormoon via het bloed terecht komt en dus waarschijnlijk een effect uitoefent. Tot dusver zijn er experimenten uitgevoerd die er op wijzen dat αMSH vooral een effect heeft op de stofwisseling door binding aan de MC4R, en daarnaast ook het immuunsysteem en de afgifte van neurotransmitters beïnvloedt. Een rol binnen de primaire stress respons (het zo snel mogelijk reageren op de stressor door de cortisol afgifte te verhogen) kon tot nu toe niet worden bevestigd.

Dankwoord

Na tijdens mijn eerste stage al in aanraking te zijn gekomen met het schrijven van een publicatie (=ontelbare verbeteringen), zag ik dat AiO-schap wel zitten, en toen ik twee jaar later na nog twee stages was afgestudeerd kwam ik dan ook bij professor Wendelaar Bonga vragen of er misschien nog AiO-plaatsen waren op de afdeling Organismale Dierfysiologie. Nadat ik mijn interesse kenbaar had gemaakt voor het project waarvan een groot aantal resultaten in dit boekje beschreven staan, liet Sjoerd al tijdens mijn afstudeerrede doorschemeren dat het wel goed zat met die baan. Mogelijk was ik de laatste in de zaal die dat doorhad.

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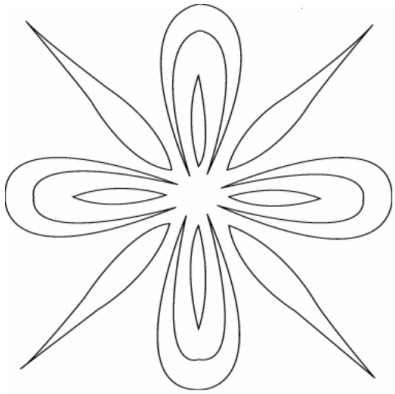
Angela



Curriculum vitae

Angela van der Salm werd geboren op maandag 10 oktober 1977 te Hoeven, N.Br. Na het behalen van het VWO diploma aan de Katholieke Scholengemeenschap Etten-Leur werd in 1995 begonnen aan de studie Biologie. Tijdens de doctoraalfase werden tussen februari 1998 en juni 2000 drie stages verricht. De eerste stage liep ze bij de afdeling Organismale Dierfysiologie onder leiding van Prof. SE Wendelaar Bonga met als dagelijkse begeleider (tegenwoordig Dr.) DT Nolan. Angela verrichte onderzoek aan de gastheer-parasiet interactie tussen de karperluis en de karper. Na iets meer dan een jaar begon de stage bij de afdeling Aquatische Oecologie onder begeleiding van (ook tegenwoordig Dr.) ECHET Lucassen en (tegenwoordig Prof.) Dr. J Roelofs. Hier onderzocht zij de relatie tussen hoge nitraat-waarden in grondwater en ijzerchlorose in cyperzegge en andere moerasplanten. Bij beide stages werd al een snufje van Ierland geroken en de derde stage verplaatste zich volledig naar University College Cork, Dpt. Of Zoology, onder begeleiding van Prof. J van Groenendaal en Dr. E Rogan. Hier werd onderzocht wat de morphometrische verschillen zijn tussen mannelijke en vrouwelijke gestreepte dolfijnen. Na het behalen van haar doctoraal begon ze in februari 2001 als Assistent in Opleiding aan het onderzoek wat is beschreven in dit proefschrift. Tijdens de aanstellingsperiode werd een bijdrage geleverd aan het Biologie onderwijs middels begeleiding van studenten voor korte en langere onderzoeksstages en het klassikaal begeleiden van studenten tijdens de practica Dierfysiologie. Delen van dit onderzoek werden gepresenteerd op nationale (KNDV congres 2001, Nijmegen) en internationale wetenschappelijke bijeenkomsten (ESCE congres 2004, Uppsala, Zweden; ISFE congres 2004, Castellon, Spanje).

Publications – Abstracts – Posters

Van der Salm, A.L., M. Martínez, G. Flik, and S.E. Wendelaar Bonga **(2004)**. Effects of husbandry conditions on the skin colour and stress response of red porgy, *Pagrus pagrus*. Aquaculture 241, pp.371-386.

Van der Salm, A.L., M. Martínez, M. Pavlidis, G. Flik, and S.E. Wendelaar Bonga **(2004)** The acute stress response of red porgy, *Pagrus pagrus* kept on a red or white background.5th International Symposium on Fish Endocrinology, 5-9 september 2004, Castellon, Spain.

Van der Salm, A.L., R. Gresnigt, G. Flik, and S.E. Wendelaar Bonga **(2004)** Colour change in tilapia can be modified by water acidification: the influence of αMSH. Uppsala J. Med. Sci. Supplement 56, pp.101 Abstracts for the 22nd Conference of European Comparative Endocrinologists

Van der Salm, A.L., D.T. Nolan and S.E. Wendelaar Bonga **(2002).** *In vitro* evidence that cortisol directly modulates stress-related responses in the skin epidermis of the rainbow trout (Onchorhynchus mykiss Walbaum). Fish Physiology & Biochemistry, 27: pp.9-18

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Van der Salm, A.L., M. Pavlidis, G. Flik and S.E. Wendelaar Bonga **(2004).** Differential release of α-Melanophore Stimulating Hormone isoforms by the pituitary gland of red porgy, *Pagrus pagrus*. Gen. Comp. Endocrinol., 135: pp.126-133

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Nolan, D.T., A.L. van der Salm and S.E. Wendelaar Bonga **(2000).** The host-parasite relationship between the rainbow trout (*Oncorhynchus mykiss*) and the ectoparasite *Argulus foliaceus* (Crustacea: Branchiura): epithelial mucous cell response, cortisol and factors which may influence parasite establishment. Contrib. Zool., 69: pp.57-63

Nolan, D.T., A.L. van der Salm and S.E. Wendelaar Bonga **(1999).** *In vitro* effects of short-term cortisol exposure on proliferation and apoptosis in the skin epidermis of rainbow trout (*Oncorhynchus mykiss* Walbaum). In: Recent Developments in Comparative Endocrinology and Neurobiology. E. Roubos, S.E. Wendelaar Bonga, H. Vaudry and A. De Loof, eds. Shaker Publishers, Maastricht, pp.161-162

Niet in dit proefschrift:

Verschillende onderzoeksprojectjes die meer licht hadden kunnen werpen op de kleurregulatie bij tilapia en red porgy, maar die dit proefschrift niet hebben gehaald en wel hierom:

- innervatie van schubben. *Schub-melanophoren kunnen ook reageren op catecholamines. Komen die daar via zenuwbanen?* Mogelijk kunnen de fluorescente probes niet doordringen tot aan de zenuwbanen. Weefselkweek van lappen huid inclusief schubben bleken ook niet te werken, het doordringen van de probe duurt veel te lang.

- rol van catecholamines bij kleurverandering in tilapia. *De snelle kleurverandering van tilapia op een nieuwe achtergrond gaat mogelijk via catecholamines. Zijn deze verhoogd na een verplaatsing naar een witte achtergrond (vs. zwart) en correleren ze met de lichaamskleur?* Kleuradaptatie van tilapia binnen een uur; ging best goed tot de zijplaat van het witte aquarium naar beneden viel en de vissen daar akelig van schrokken en ineens weer een stuk donkerder waren.

- rol van α en β adrenerge receptoren bij kleurverandering: pogingen om deze receptoren te kunnen isoleren en karakteriseren middels moleculaire technieken hadden geen succes. Gelukkig lijken de melanocortine receptoren enorm op elkaar in allerlei diersoorten en heb ik die dus wel kunnen vinden ☺

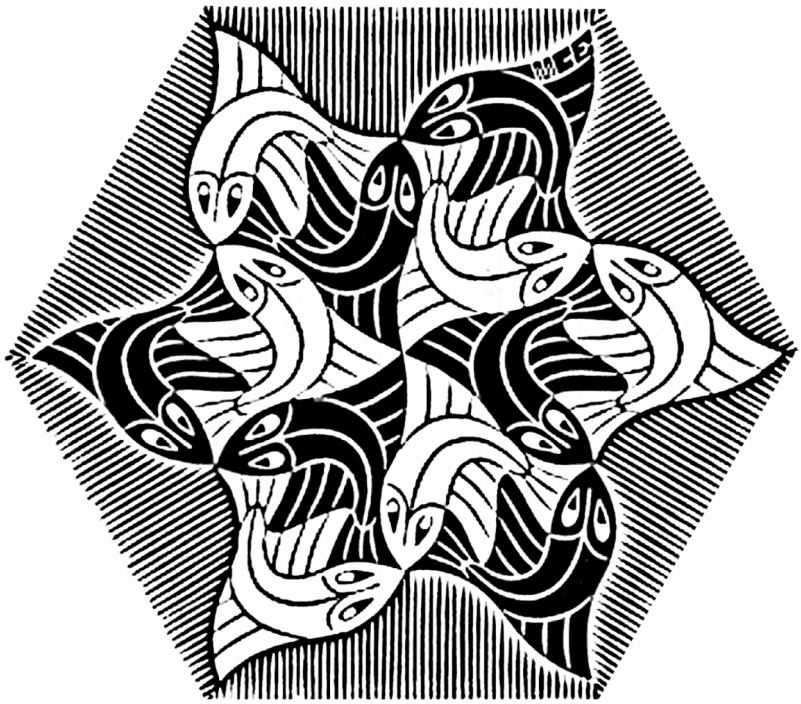
- studies op melanophoren van red porgy. Binnen een Europees project heeft iedere partner zijn of haar eigen taak en deze taak lag bij een ander. In dit proefschrift dus alleen resultaten behaald bij tilapia.

- het inspuiten van αMSH in de buikholte van red porgy en in spier van tilapia. *Als de concentratie circulerend αMSH kunstmatig omhoog wordt gebracht, worden de vissen daar dan ook donkerder van?* In de buik is het na twee dagen uit het lichaam verdwenen en in de spier duurt het langer dan een dag om in het lichaam opgenomen te worden. Kleurverandering was niet duidelijk aan te tonen.

Stellingen behorend bij het proefschrift:

αMSH in fish

Functions in stress responses and skin colour change



Angela van der Salm

Stellingen

1] De verschillen tussen in vitro effecten van αMSH en in vivo functies wijzen op een geringe fysiologische betrokkenheid van αMSH bij de pigmentatie-verandering van de huid in red porgy en tilapia.
Dit proefschrift

2] Onderzoekers aan hormonen worstelen vaak met het probleem dat bijna geen enkel hormoon een specifieke eenzijdige functie heeft.
Vele proefschriften

2A] Ook αMSH behoort tot die groep hormonen.
Dit proefschrift

3] Ondanks het inzicht van dr. Fujii om het zoogdier-systeem te gebruiken voor het classificeren van melanocortine-receptoren, heeft hij dit niet doorgevoerd voor het classificeren van αMSH-receptoren. Zijn suggestie om een α-MC-R en een β-MC-R is dan ook nooit opgevolgd.
Fujii (2000),Pigment Cell Research 13, pp300-319

4] In homeotherme dieren zijn variaties in huids- of vachtkleur verbonden met een mutatie in het MC1-receptorgen. De flexibiliteit van pigmentcellen in poikilotherme dieren wijst erop dat soortgelijke mutaties, als ze al bestaan, veel minder effect zullen hebben op de kleur van de huid.
Rees (2003), Annual Reviews of Genetics 37, pp.67-90

5] De waarden van "L*", "chroma" en "hue" in de red porgy blijken interessant genoeg overeen te komen met de waarden gevonden bij mensen. Schotten (rood haar, sproeten, lichte huid) hebben de hoogste L-waarde en hue en de laagste chroma. Helaas voor hen is de huidskleur van mensen veel minder veranderlijk dan van vissen dus een gebruinde Schot zul je niet snel zien.
Trujillo (1996), Forensic Science International 81, pp.1-10

6] Als het gat in de ozonlaag nog groter wordt, wil er straks toch niemand meer bruin worden in de zon.

7] Moleculaire biologie hoef je niet te begrijpen om het toe te kunnen passen.
Klaas, toen hij 3e-jaars student was

8] Wetenschap is georganiseerde kennis. Wijsheid is een georganiseerd leven.
Immanuel Kant

9] Een wetenschapper kijkt door het sleutelgat van de natuur om erachter te komen hoe ze in elkaar zit.
Jacques Cousteau

10] Hope is the destination that we seek.
Love is the road that leads to hope.
Courage is the motor that drives us.
We travel out of darkness into faith.
Dean Koontz, Book of Counted Sorrows

In this thesis, the functions of the hormone αMSH (α-melanophore stimulating hormone) in skin colour control and in the stress response have been investigated in two species of fish, red porgy (*Pagrus pagrus*) and tilapia (*Oreochromis mossambicus*). The darkness of the skin can be enhanced by adaptation to a black background in both species, yet this is not mediated by increases in circulating αMSH levels. The process of background adaptation can be compromised by acid water-induced stress in tilapia, but was not affected by a variety of stressors in red porgy. Acute stress can induce a rise in plasma αMSH levels in red porgy and chronic low water pH can increase plasma αMSH in tilapia. Isolated scale melanophores do show a dispersing response after stimulation with the three isoforms of αMSH. In summary, αMSH can cause fish skin to darken but this function is reduced *in vivo*, indicating a role for other skin colour controlling agents (MCH, catecholamines). Some types of stressors do cause plasma αMSH levels to rise while others failed to do so. A function in the stress response may be more related to metabolic regulation rather than to a direct function in the release of catecholamines or cortisol.

Photo courtesy of Klaas Medendorp

αMSH in fish - Functions in stress responses and skin colour change

Angela van der Salm